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Immunosuppressive Drug Interactions and Resistance in Mononuclear Cells from Renal Transplant Patients

by

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DECLARATION

I confirm that my thesis has been prepared in accordance with the University's guidelines on the presentation of a research thesis (October 1999). Part of this thesis has been published in a peer-reviewed international journal (Reference: Transpl Int 2000; **13**: 276-284). This included work on lymphocyte sensitivities to the immunosuppressant drugs cyclosporin A and FK506 and the subsequent investigation of resistant mechanisms.

The thesis is my own work apart from preliminary experiments on lymphocyte sensitivity that were performed by Ms. Emma Smythe. I assisted in the recruitment of patients and data analysis during this part of the work. The rest of the thesis, including investigation of drug resistance and investigation of drug interactions is entirely my own work.

I can confirm that the thesis has not been submitted for a degree at another university.

SUMMARY

Existing anti-rejection drug regimes are inadequate since patients receive drugs despite serious side effects and poor response. New drugs are being developed which ultimately may allow for prescribing of rational, patient-specific immunosuppressive drug protocols. During this thesis the investigation of lymphocyte responses from renal transplant recipients to the immunosuppressant drugs Cyclosporin A (Cy A), FK506 and SDZ RAD were explored to understand the variation in sensitivity of lymphocytes to Cy A and FK506, the development of drug resistance, including resistance mechanisms, and the interactions between FK506 and SDZ RAD.

Cy A and FK506 are substrates for P-glycoprotein (P-gp), the product of the multidrug-resistance (MDR1) gene in man. A hypothesis established during this thesis was that P-gp dependent mechanisms explain variations in lymphocyte sensitivities to Cy A and FK506.

Lymphocytes from renal transplant recipients were assessed for their sensitivity to Cy A and FK506 and subsequently for P-gp expression and functional activity by flow cytometry. In further lymphocyte cultures the effect of the specific P-gp inhibitor, PSC 833 on sensitivity was investigated. Finally, the effects of the combination of FK506 and SDZ RAD in lymphocyte cultures were analysed.

Results demonstrate a wide range in lymphocyte sensitivity to both Cy A and FK506, with the development of selective resistance to the drug used for treatment. All patients demonstrated P-gp functional activity but P-gp expression was not demonstrable. P-gp function did not account for the variation in lymphocyte sensitivity. There was no evidence of antagonism of effect of SDZ RAD in combination with FK506.

In conclusion, these results suggest that non-P-gp mechanisms account for variations in lymphocyte sensitivity to Cy A and FK506. Combination therapy with SDZ RAD and FK506 is unlikely to be antagonistic in future treatment protocols.

ABBREVIATIONS

ABC	ATPase Binding Cassette
ADR	Adriamycin
APC	Antigen Presenting Cells
AST	American Society of Transplantation
ASTP	American Society of Transplant Physicians
ATP	Adenosine Triphosphate
AUC	Area Under the Curve (refers to concentration-time curve obtained in drug absorption profiling)
AZA	Azathioprine
β-ME	β-mercaptoethanol
C-	Chromosome-
calcein AM	calcein-acetoxymethyl ester
CAN	Chronic Allograft Nephropathy
CD	Cluster of Differentiation (defines cell lineage markers: for example, CD4+ or CD8+ cells)
Ci	Curie
C-0	Trough or pre-dose level of a drug
Cmax	Highest level of drug reached following ingestion
CO_2	Carbon dioxide
CIT	Cold Ischaemia Time
CMV	Cytomegalovirus
CN	Calcineurin
Con A	Concanavalin A

CTL	Cytotoxic T Lymphocytes
Cy A	Cyclosporin A (Sandimmun(R)\ Neoral(R))
DMSO	Dimethylsulphoxide
cDNA	complimentary Deoxyribonucleic Acid
DNR	Daunorubicin
DTH	Delayed-Type Hypersensitivity
DR	Refers to the Class II Region of The MHC
4E3	Monoclonal antibody against external epitope of P-gp
EDTA	Ethylene-diamine-tetra-acetic acid
ESRF	End-Stage Renal Failure
FACS	Fluorescence-activated cell sorter (flow cytometer)
FITC-	Fluorescein isothiocyanate
FK506	Generic name for Tacrolimus (Tac or Prograf(R))
FKBP	FK506 binding proteins
FRAP	FKBP12-rapamycin-associated protein
GFR	Glomerular Filtration Rate
HLA	Human Leucocyte Antigens (The phenotype in man of the Major Histocompatibility Complex)
ID_{50}	Inhibitory dose 50% (the concentration of drug that inhibits cell proliferation by half)
IDDM	Insulin Dependent Diabetes Mellitus
Ig	Immunoglobulin
IL-	Interleukin- (for example IL-2)

IL-2R	IL-2 Receptor
ISH	in situ hybridization
kD	Kilo Dalton
LRP	Lung resistance protein
M	Molar
Mab	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MDR	Multidrug-resistance
MDR1	Multidrug-resistance gene1 in man
MFI	Mean fluorescent Intensity
MLR	Mixed Lymphocyte Reaction
6-MP	6-mercaptopurine
MRK	MRK16; Monoclonal antibody against external epitope of P-gp
MRP	Multidrug-Resistance-Associated Protein
μ	micro
mins	minutes
mls	millilitres
mTOR	Mammalian Target of Rapamycin
NBD	Nucleotide-Binding Domains
NF-AT	Nuclear Factors of Activated T cells
NK cells	Natural Killer cells
nm	nanometre
Pt.No.	Patient number

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PE-	Phycoerythrin-
P-gp	P-glycoprotein-170
PHA	Phytohaemagglutinin
PMA	Phorbol Myristate Acetate
PSC 833 (Valspodar®)	Generic name of cyclosporin analogue
'R'	Responder cells
RAFT	The rapamycin and FKBP-target
RAPA	Rapamycin (Sirolimus)
mRNA	Messenger Ribonucleic Acid
rpm	revolutions per minute
R-123	Rhodamine-123
RT/PCR	Reverse transcriptase/polymerase chain reaction
RPMI 1640	Roswell Park Memorial Institute 1640 medium
'S'	Stimulator cells
SDW	Sterile Distilled Water
SD	Standard Deviation
SDZ RAD	Generic name for the rapamycin analogue, Everolimus (RAD or Certican®)
SI	Stimulator Index
TGF-β	Transforming Growth Factor β
Th1	T helper cells, type 1

TMD	Transmembrane Domains
^{3}H-	Tritiated-
$T_{\frac{1}{2}}$	Half-life (referring to a drug, or in terms of transplanted organs, the time taken for half of all allografts to fail)
UCLA	University of California at Los Angeles
UK	United Kingdom
UNOS	United Network for Organ Sharing
USA or US	United States of America

CHAPTER 1

INTRODUCTION

Preface

Allograft recipients have to take powerful drugs to minimise rejection of the graft. In clinical transplantation there is a delicate balance of anti-rejection drug efficacy and serious side effects including hypertension, nephrotoxicity, increased risk of cancer and infections. New drugs are being developed and introduced into clinical practice and until means of inducing allograft tolerance is introduced into the clinic the treatment goals remain to use these drug combinations to improve efficacy and reduce side effects. For example combining drugs at lower than the normal therapeutic levels may lead to a degree of synergy with improved efficacy and less side effects. Existing drug regimes are inadequate since patients will continue to receive drugs despite marked side effects and poor response. This balance may be improved by tailoring immunosuppressive therapy protocols to individual patients.

The idea behind the work described in this thesis was to develop a means of studying *in vitro* patient lymphocyte responses to immunosuppressive drugs in order to understand their mode of action, mechanisms of resistance, and interactions between them. This may allow for future prescribing of rational, patient-specific immunosuppressive drug protocols.

1.1 Renal transplantation

1.1.1 Introduction

Transplantation is the process of taking cells, tissues, or organs, called a graft, from one individual and placing them into a different individual. A graft transplanted between two genetically different individuals is called an allograft.

This thesis is concerned with kidney transplantation in the form of whole organ kidney (renal) allografts.

1.1.2 Historical perspective

This historical overview of transplantation is derived from two texts (Kuss and Bourget, 1992 and Brent, 1996). The earliest recorded attempts at organ transplantation date back thousands of years. However, it was in 1902 that Ullmann performed a successful renal autotransplant (a graft transplanted from one individual to the same individual) in a dog. Carrel, in 1912, showed that renal autografts survived indefinitely, but that renal allografts rapidly cease to function. In the 1940s experiments by Medawar into failing skin grafts described an inflammatory reaction called rejection, which was shown to be a form of specific immunity. Clinical transplantation did not become a reality until 1954. Murray and colleagues performed the first successful kidney transplantation between monozygotic twins in Boston, USA. The success of this pioneering work was

based on a number of earlier observations from animal and human experiments. They quote ..."several experimental observations which made success between identical twins seem likely and justified the removal of a normal kidney from a healthy donor.

(1) Immunological and genetic similarity apparently accounts for the permanent survival of skin homografts between identical twins.

(2) When skin or kidney homografts are carried out between antigenically dissimilar individuals the early function and the histological picture of rejection of each appear similar.(3) Skin and kidney homografts possess a common antigen that can sensitise a recipient to a subsequent homograft of either tissue from the same donor. This further suggests that skin and kidney grafts might behave similarly".

In 1959, Dameshek and Schwartz used 6-mercaptopurine (6-MP) in place of irradiation to precondition patients for bone marrow transplantation. This work was developed by Calne with the introduction of a safer derivative of 6-MP called azathioprine (AZA) into renal transplantation. By 1963, maintenance AZA and corticosteroids became the standard regimen for renal transplantation. Allograft survival with these treatment protocols was approximately 40% at 12 months. In the late 1970s to early 1980s Cyclosporin A (Cy A) was introduced and has been the mainstay of immunosuppressive regimes in combination with AZA and corticosteroids. Cy A based protocols led to fewer episodes of acute rejection episodes and improved allograft survival at 12 months to 80-90%. FK506 (Tacrolimus) has been introduced during the 1990s and other experimental drugs, including rapamycin (Sirolimus, RAPA), are being developed. Concurrently there

has been marked progress on the development of use of biological agents to suppress the immune response to an allograft in human transplant recipients. All these advancements have led to increases in allograft and patient survival with one-year graft survival of approximately 90% being the rule rather than the exception (Gjertson, 1994). By contrast, long-term graft survival remains a major problem. This has served to focus attention to the longer-term outcome in an attempt to manage patients to obtain optimal long-term graft survival while minimising patient morbidity. The search continues, therefore, for the ideal immunosuppressive agent, namely one that selectively inhibits alloantigen immune responses, prevents chronic allograft rejection, and is free of major side effects.

1.1.3 Current perspective

Renal transplantation is the treatment of choice for patients with end-stage renal failure (ESRF). The supply of donor organs, which averages 30/million population per year in the United Kingdom, is greatly outstripped by demand, which is a minimum of 48/million population per year. The number of patients on the active list for renal transplantation in the UK has increased from 3800 in 1993 to 4908 in 2000 (UK Transplant, 2001). Only 28% of patients with ESRF were on the waiting list in 1998 (The UK Renal Registry 2000).

Not all patients receiving dialysis are suitable for transplantation. In the United Kingdom formal acceptance criteria onto a transplant waiting list have not been established, although patients with diabetes, severe vascular disease and a history of malignancy have a lower likelihood of receiving a transplant since their reduced life span would lead to death with a functioning graft. Indeed, despite these criteria death with a functioning graft remains the most common cause of graft loss (Prommool *et al.*, 2000). The situation is further complicated by the observation that a number of the patients in these high-risk groups would in fact have an improved life expectancy with a transplant compared to dialysis treatment (Port *et al.*, 1993).

Since 1975, 1 year graft survival rates for primary cadaveric renal transplants in the USA have risen from 50 to almost 90% [based on data collected by University of California at Los Angeles (UCLA) and United Network for Organ Sharing

(UNOS) Kidney Transplant Registries (Gjertson, 1994)]. Much of this improvement has been attributed to the introduction of Cy A into treatment protocols. However, survival curves indicated that the long term loss rate of transplanted kidneys remained fairly stable until the early 1990s with the time taken for half the grafts to fail (half-life, $t_{1/2)}$) remaining between 8.5 to 9 years (See Figure 1.1).

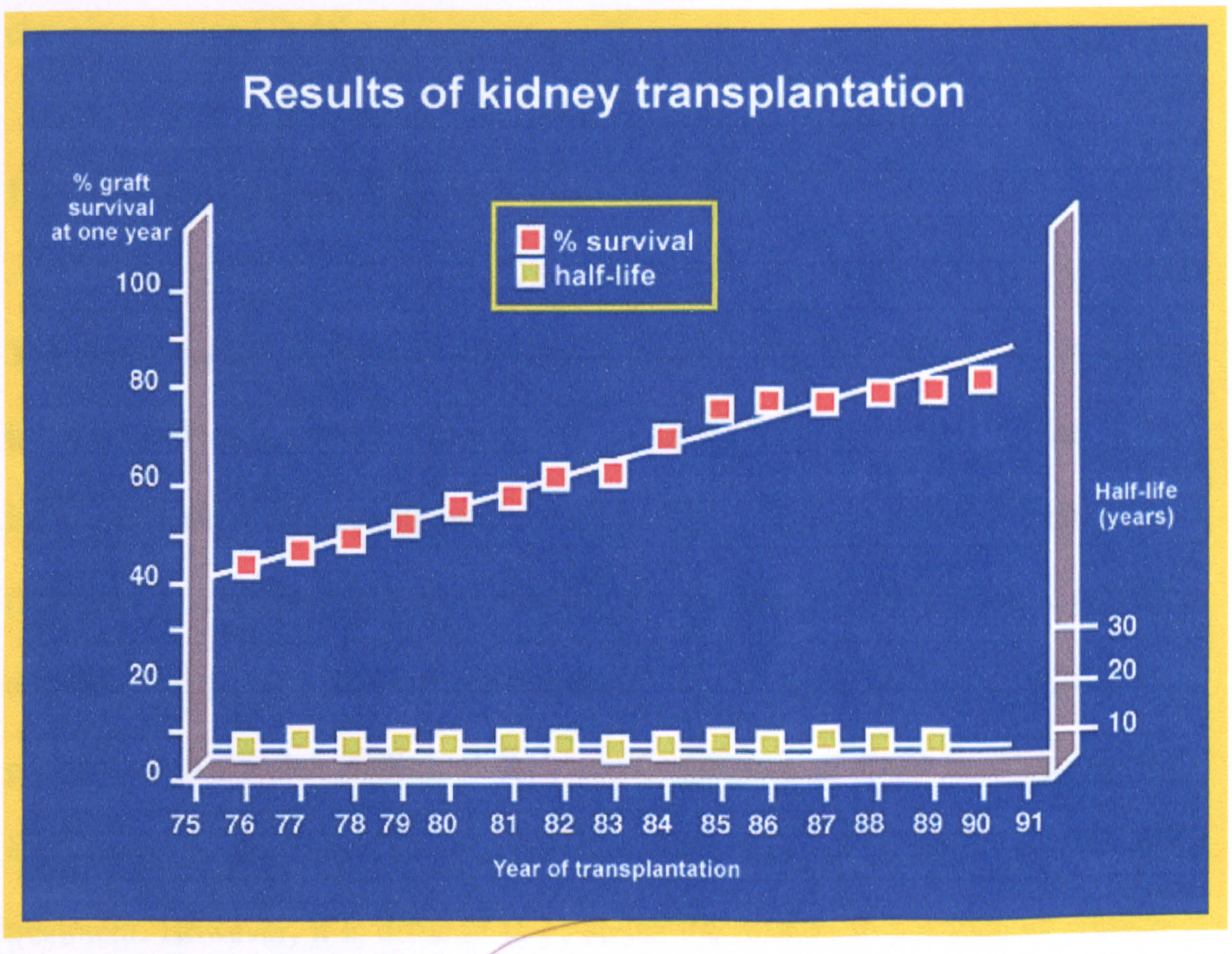


Figure 1.1 Results of kidney transplantation. The graph shows the increase in allograft one-year survival from 40% during 1975 to 80% in 1990. There was little impact upon allograft half-life during this period (Figure taken from The Atlas of Transplantation, Roche. Based on original data from Gjertson, 1991).

Thus, the initial advantage of Cy A treatment diminished beyond 1 year. The half-life for allografts has improved, both for Cy A treated patients and those patients treated with FK506, with data from UNOS suggesting a half-life of 11-12 years during the mid-1990s (Hariharan *et al*., 1996). More recently the projected half-

life for grafts from living donors has increased steadily from 12.7 to 21.6 years, and that for cadaveric grafts has increased from 7.9 to 13.8 years (Hariharan *et al.*, 2000). After censoring of data for patients who died with functioning grafts, the projected half-life for grafts from living donors has increased from 16.9 years to 35.9 years, and that for cadaveric grafts has increased from 11 to 19.5 years (Hariharan *et al.*, 2000). If death with a functioning graft is removed from the analysis then the predominant cause of graft dysfunction and ultimate graft loss is chronic rejection (Jindal and Hariharan, 1999).

1.1.4 Chronic Allograft Nephropathy

The mechanisms of chronic rejection are being clarified and since multiple immune and non-immune factors play a part in chronic rejection, the more inclusive term 'chronic allograft nephropathy' (CAN) has been introduced (Halloran *et al.*, 1999a). This condition is manifested clinically by a gradual decrease in renal function accompanied by hypertension and low-grade proteinuria. An understanding of the causative factors of CAN is of paramount importance to the transplant community, since there is currently no specific treatment. However, it is difficult to establish the relative importance of putative causative factors due to the heterogeneity of medical practice between transplant centres both in the UK and throughout the world. For example, there is marked variation in donor management, surgical technique, tissue-type matching, recipient selection and drug protocols. Notwithstanding, univariate and multivariate analyses have been performed on donor and recipient variables on data from UNOS (Cecka *et al.*, 1992, Gjertson, 1994 and Hariharan *et al*, 2000).

The conclusions from these studies are that the causes of CAN are multi-factorial and can be broadly divided into two groups, namely, immunological and non-immunological. The major immunological factor contributing to CAN is one or more episodes of acute rejection within the first 12 months following transplantation. Among patients who had an episode of clinical acute rejection, the projected half-life of cadaveric transplants was 7 years in 1988 and 8.8 years in 1995, an increase of 25%. In contrast, the projected half-life for cadaveric transplants in patients who did not have an episode of acute rejection in the first 12 months following transplantation was 8.8 years in 1988 and 17.9 years in 1995 (Hariharan *et al.*, 2000). Risk factors for development of acute rejection episodes and subsequent CAN include HLA-DR mismatches and highly sensitised individuals manifested by a high percentage of panel-reactive antibodies in their serum (Gjertson, 1994 and Connolly *et al.*, 1996). Further discussion of HLA follows in section 1.2.2.1. Apart from clinical acute rejection, patients may have sub-clinical rejection that causes ongoing immunological injury (Rush *et al.*, 1995).

There are a number of non-immunological factors that contribute to CAN. For example, a prolonged cold ischaemia time (the time the donor organ remains on ice *ex vivo*) of greater than 30 hours is associated with peri-transplantational injury and delayed graft function (Gjertson, 1994, and reviewed by Pascual *et al.*, 2002). Analyses of the relative importance of quality of donor kidney, increasing donor age, mode of donor death, racial and socio-economic factors, and exposure to

cytomegalovirus infection can be found in a number of studies (Gjertson, 1994, Morris, 1994 and Cole *et al.*, 1995). Specifically, in the USA, the projected half-life of a kidney in a black recipient is 6 years compared to an Asian recipient of 17 years (Gjertson, 1994). This disparity of allograft survival raises the possibility of genetic influences on allograft survival as well as those relating to socio-economics and compliance. The studies also report the use of organs from older cadaveric donors. Kidneys from such donors have a lower survival rate than those from younger cadaveric donors (Gjertson, 1994). Due to limited availability, the proportion of grafts from older cadaveric donors increased from 10.4 to 18.2 percent from 1988 to 1996 (Hariharan *et al.*, 2000).

1.1.5 Impact of immunosuppressive drugs in clinical transplantation

As discussed in section 1.1.2 the mainstay of anti-rejection regimens have included corticosteroids, in the form of prednisolone, and azathioprine. These drugs in combination with either Cy A or FK506 are the predominant regimes comprising 'triple therapy' in use in UK transplant centres. Treatment with prednisolone in high dose or prolonged treatment is associated with a number of non-immune side effects such as development of diabetes mellitus, osteoporosis, thinning of the skin and truncal obesity (British National Formulary, 2001). Bone marrow suppression is the major dose limiting side effect of treatment with azathioprine (British National Formulary, September 2001).

The rationale for the introduction of new compounds into clinical transplantation is to increase the efficacy of the drug regimes and reduce drug toxicity. Some side

effects (infections and malignancy) reflect lack of adequate immune function and are inherent in suppressing the host immune response. These side effects include infections, such as pneumocystis pneumonia, cytomegalovirus, and certain malignancies, such as skin cancer and lymphoma. A retrospective questionnaire study from Leeds, UK investigated 918 patients transplanted from 1967-1991 (London *et al.*, 1995). The study demonstrated 80 different tumours in 70 patients: 42 in skin, 12 of urogenital origin, 5 B cell lymphomas plus a number of miscellaneous tumours. In an age and sex-matched normal population after 20 years, a 6% prevalence of cancer was observed, compared with a prevalence of 13.4% at 10 years and 40% at 20 years in the transplant population (London *et al.*, 1995). Recent data has shown that the prevalence of non-melanotic skin cancer in transplant recipients at 10 years is almost 30% in the UK and 86% in Queensland, Australia (Ramsay *et al.*, 2001). These data suggest that patients receiving immunosuppressive drugs require lifelong surveillance for malignancy.

1.1.5.1 Cyclosporin A

Following the introduction of Cyclosporin A (Cy A) in the 1980s there was a dramatic increase in 1-year allograft survival due to a reduction in the number of acute rejection episodes (Merion *et al.*, 1984). However, a number of important side effects have been observed. Nephrotoxicity was soon apparent in the early clinical trials (Calne *et al.*, 1978). This required limiting the drug to those patients who were passing adequate volume of urine (Calne *et al.*, 1978 and 1979). It is now practice to use much lower doses of Cy A in immunosuppression drug protocols in an effort to avoid acute toxicity. However, chronic Cy A

nephrotoxicity remains a major clinical problem (Griffiths *et al.*, 1996). The accompanying decreased glomerular filtration rate (GFR) and raised serum creatinine levels can lead to diagnostic confusion with acute and chronic rejection. There are no diagnostic morphological features on biopsy in acute toxicity, but lesions of chronic nephrotoxicity consist of the vascular changes of arteriolar hyalinisation and glomerular ischaemia leading to glomerular occlusion, and tubulointerstitial changes producing interstitial fibrosis and tubular atrophy (Griffiths *et al.*, 1996, Benigni *et al.*, 1999). The aetiology of Cy A induced nephrotoxicity is unclear. It has been postulated that sustained glomerular arteriolar vasoconstriction eventually promotes structural alteration and occlusion of glomerular vessels (Myers *et al.*, 1988). Although renal hypoperfusion, as demonstrated by low renal blood flow has been demonstrated following administration of Cy A, the tubulointerstitial injury may be a separate entity that develops independently of the vasoactive effects of the drug (Benigni *et al.*, 1999). Renal biopsy material obtained from patients with histological features of nephrotoxicity revealed frequent tubulointerstitial lesions and few glomerular lesions. Intense staining of tubular epithelial cells for the vasoactive and chemotactic mediators, endothelin-1, RANTES and monocyte chemoattractant protein-1 mRNAs, was demonstrated and it is the local production of these molecules that may lead to inflammation and fibrosis (Benigni *et al.*, 1999).

1.1.5.2 FK506 (Tacrolimus)

FK506 is indicated for use as primary immunosuppression in liver and kidney allograft recipients and liver and kidney allograft rejection resistant to

conventional immunosuppressive regimes. However, the role of FK506 in clinical organ transplantation continues to evolve. Much of the early promise with FK506 has been reported in liver transplantation with a clear benefit over Cy A in the prevention of acute and chronic allograft rejection (The European and US FK506 Multicenter Liver Study Groups, 1994). Preliminary clinical experience following renal transplantation suggests that primary prophylaxis with FK506 results in graft and patient survival rates that are equivalent to those achieved with conventional Cy A based therapy with an overall graft survival of 85-90% at one year and one-year patient survival of 94% (Schleibner *et al.*, 1995). The incidence of acute rejection episodes was 48% (Shapiro *et al.*, 1995). Studies directly comparing the effects of FK506 with Cy A have been reported (Pirsch *et al.*, 1997 and Mayer *et al.*, 1997). The American study reported by Pirsch assessed over 400 patients that were randomised to receive either FK506 of Cy A after cadaveric renal transplantation. Again one year patient survival rates were comparable. There was a slight improvement in one year graft survival rates with FK506 compared to Cy A [91.2% and 87.9% (p=0.289)], and a significant reduction in the incidence of biopsy proven acute rejection episodes in the FK506 group (30.7%) compared with the Cy A treated group (46.4%). Similar results were obtained in the European Tacrolimus Multicenter Renal Study Group where 448 renal transplant patients were recruited and assigned to receive triple-drug therapy consisting of FK506 or Cy A with AZA and low dose corticosteroids (Mayer *et al.*, 1997). Preliminary data also suggest that long-term survival as estimated from $t_{1/2}$ may be improved with FK506 based regimes (Gjertson *et al.*, 1995). These studies have confirmed that there are a number of serious side effects associated with FK506. Nephrotoxicity is common with FK506 treatment with incidence at one year

reported between 45-57.6% (Pirsch *et al.*, 1997 and Vincenti *et al.*, 1996). The incidence of hypertension was similar to that with Cy A and tremor was more often seen with FK506 (Pirsch *et al.*, 1997. Hirsutism, gingivitis and gum hyperplasia were negligible compared to that obtained with Cy A (Mayer *et al.*, 1997). The overall infection rates were comparable between treatment groups, as was the incidence of opportunistic infections (Mayer *et al.*, 1997). Hyperlipidaemia was lower in the FK506 treated group compared to the Cy A treated group (Mayer *et al.*, 1997). Of particular concern, however, is the development of *de novo* insulin dependent diabetes mellitus (IDDM) in the FK506 Groups. The initial incidence of post transplant diabetes mellitus, which was defined as the use of insulin for 30 days or longer in patients who had no history of IDDM or non-insulin dependent diabetes mellitus was 19.9% in FK506 treated patients and 4% in Cy A treated patients (Pirsch *et al.*, 1997). At 12 months, however, the proportion of FK506 treated patients receiving insulin was 5.5% and withdrawal of treatment with FK506 due to diabetes mellitus occurred in 1.5% of patients (Mayer *et al.*, 1997).

In addition to the evolving value of FK506 as a primary immunosuppressant in treatment schedules the use of FK506 in salvaging allografts has been investigated (Jordan *et al.*, 1994 and 1997, Eberhard *et al.*, 1996). Resistant acute allograft rejection under Cy A based immunosuppression remains a considerable problem despite the use of anti-lymphocyte preparations. In 1994, Jordan reported a series of 77 patients with refractory acute rejection on baseline Cy A therapy that were converted to FK506 and graft salvage was achieved in 74% of cases (Jordan *et al.*, 1994). The 1997 report included a further 92 patients with similar results (Jordan

et al., 1997). The indications for conversion to FK506 were ongoing, biopsy confirmed rejection in all patients. The median interval to conversion was two months after transplantation and all patients had received treatment with high dose corticosteroids and 85% of the patients also receiving a course of an anti-lymphocyte preparation in an effort to ameliorate the rejection episode.

Indeed, the initial stimulus to this present work was the observation that FK506 could be effective in treating patients in whom Cy A had failed. It was surmised that sensitivity to the drugs was i) independent and ii) that resistance to these drugs could develop.

1.1.5.3 Rapamycin (Sirolimus/RAPA)

Rapamycin has recently been introduced into clinical transplantation. Rapamycin is a powerful immunosuppressant that is 10-100 fold more potent than Cy A in preventing rejection of vascularised grafts in animal models and is also effective in alleviating ongoing rejection of kidney, pancreas and heart allografts in the rat (Stepkowski *et al.*, 1991, Granger *et al.*, 1995). The main clinical data come from three major studies. In 11 European centres, first cadaveric renal allograft recipients were randomised to receive either Cy A or rapamycin as part of a triple regime with corticosteroids and AZA (Groth *et al.*, 1999). At 12 months, graft survival was excellent in both groups (98% and 90% respectively) as was patient survival (100% and 98%) and incidence of acute rejection (41% and 38%). Rapamycin did not cause nephrotoxicity, gingival hyperplasia or tremor in contrast to Cy A. Opportunistic infection rates were comparable although there

was a higher incidence of pneumonia. Patients treated with rapamycin developed a higher incidence of thromboeytopenia and hyperlipidaemia (Groth *et al.*, 1999). The Rapamune US Study Group reported a large prospective, multicentre, randomised trial comparing rapamycin to azathioprine in Cy A and prednisolone based regimes (Kahan *et al.*, 2000). The results show that the severity and incidence of acute rejection episodes up to six months following renal transplantation were significantly lower in the rapamycin based regimes compared to azathioprine-based treatment (acute rejection rates for rapamycin at low dose of 16.9%; rapamycin at higher dose, 12.0%, and azathioprine, 29.8%: p< 0.001). Patient and graft survival across the three groups were similar and all greater than 90% at 12 months. Adverse events in the rapamycin treated patients were divided into those associated with an exacerbation of Cy A-related toxic effects such as hypercholesterolaemia, hypertension, headache, hirsutism, hyperkalaemia and acne, and those related specifically to rapamycin, such as hypertriglyceridaemia, thrombocytopenia and lymphocoele formation.

Finally, the Rapamune Global Study Group performed a placebo-controlled trial of rapamycin when added to a regimen containing Cy A and prednisolone (MacDonald *et al.*, 2001). The study confirms the efficacy of rapamycin at reducing acute rejection in renal transplant recipients.

Rapamycin is non-nephrotoxic (Groth *et al.*, 1999, Kahan *et al.*, 2000 and Macdonald *et al.*, 2001). However, the data from the Rapamune US Study Group reveal that mean serum creatinine concentrations are higher at 12 months in the rapamycin treated patients compared to the azathioprine group. This may have

been brought about by increased exposure to Cy A by a pharmacokinetic interaction of Cy A with rapamycin (Kahan *et al.*, 2000).

1.1.5.4 SDZ RAD (Everolimus\ RAD \Certican)

SDZ RAD is a rapamycin analogue that has recently been introduced into clinical trials and is being evaluated for its potential synergistic activity with Cy A (Schuurman *et al.*, 1997). To date outcome data with SDZ RAD is limited. A phase I study of SDZ RAD in renal transplant recipients has recently been reported (Kahan *et al*, 1999). This randomised, blinded, placebo-controlled study assessed the safety profile and pharmacokinetics of a 4-week course of once daily, sequential increasing doses of SDZ RAD. The pharmacokinetic parameters of SDZ RAD showed dose proportionality, a good correlation with trough level and area under the curve (AUC), and shorter half-life than rapamycin with the attainment of steady state levels by day 4. Studies in de novo liver transplant recipients and de novo renal transplant recipients followed for 12 months revealed no differential effects on Cy A pharmacokinetics or dosing (Levy *et al.*, 2001 and Kovarik *et al.*, 2001). However, a preliminary report to the American Society of Transplantation (AST) 2001 suggests that co-administration of SDZ RAD with Cy A increases the bioavailability of SDZ RAD (Kalbag *et al.*, 2001). Phase III trials from the RAD 251 Study Group and the RAD International Liver Study Group are ongoing.

1.1.6 Developing future anti-rejection protocols

In summary, the side effects of FK506 and Cy A, particularly nephrotoxicity, are a limitation to the greater use of these drugs. Rapamycin is being introduced into clinical transplantation since it is both efficacious and non-nephrotoxic. The combination of rapamycin or SDZ RAD with Cy A is being explored for evidence of synergy.

One of the interests of the current study has been the investigation of the rapamycin analogue, SDZ RAD and the interaction of this drug with FK506 in *in vitro* systems of lymphocyte proliferation. There are a number of reasons for this to be a useful area of study. Very briefly, *in vitro* work on murine cells suggested that the two drugs were mutually antagonistic (Dumont *et al.*, 1990a). This observation suggests that both drugs share the same intracellular binding site. However, further studies in experimental animal models suggested no antagonism, but additive or even synergistic effects (Vu *et al.*, 1997). It is therefore of interest to examine this polarisation of effect in lymphocytes from renal transplant recipients.

1.2 The immune response to an allograft

1.2.1 Introduction

The immune system has developed to form a highly specialised and tightly regulated series of responses that function to recognise self from non-self and to respond to tissue injury. T cells are crucial for the immune response to allografts. This has been demonstrated by the observation that in murine models that lack T cells cannot reject allografts (Bolton *et al.*, 1989). The central role of the T cell and the consequent targeting of T cell activation by the currently available immunosuppressant drugs will be discussed.

1.2.2 The T cell

1.2.2.1 Antigen presentation

T cells can respond to processed antigen presented on antigen presenting cells (APC) bound in the antigen-binding groove of the major histocompatibility complex (MHC) molecules, designated HLA in humans. The MHC loci encode polymorphic cell surface glycoproteins, referred to as Class I and Class II molecules. In humans the MHC Class I molecules are expressed on the surface of nucleated cells and consist of HLA-A, -B, -C. Cell surface Class II molecules are HLA-DR, -DP, -DQ, found primarily on APC. Since the alleles at all these loci are highly polymorphic and are co-dominantly expressed there will be multiple

HLA disparities in unrelated individuals, which would lead to 'histo'-or tissue-incompatibility should an allograft be necessary.

The immune response to 'standard' non-allo-antigens such as bacteria, toxins, viruses and tumour proteins, is self-MHC restricted i.e. T cells are only activated by peptides bound to self-MHC molecules (Zinkernagel and Doherty 1974). Mature T cells are subdivided based on expression of either the CD4 or CD8 glycoproteins. CD4+ cells are MHC class II-restricted, and CD8+ T cells are MHC I-restricted in terms of antigen recognition. This implies that recognition of exogenous antigens processed by APC is almost entirely dependent on CD4+ cells. The peptide antigen/MHC complex can interact with the T cell via the T cell receptor (TCR) for antigen (Garboczi *et al.*, 1996 and Garcia *et al.*, 1996).

1.2.2.2 T cell recognition of allografts

The principle targets of the immune response to allografts are the MHC molecules themselves, and T cell recognition of the allo-MHC is the primary and central event that initiates acute allograft rejection in grafts that are mismatched for MHC (Krensky *et al.*, 1990). The normal T cell repertoire contains a high frequency (1-10%) of total T cells that are capable of responding to allo-MHC molecules. This is 100-1000 times as high as a standard response to a non-self antigen presented in association with self-MHC (Krensky and Clayberger, 1993). Two major models of allorecognition have been proposed (reviewed by Turvey and Wood, 1999). In the direct pathway allorecognition involves recognition of foreign MHC molecules expressed on the surface of donor cells. Since T cells are normally 'self-

restricted', in that they only react to self-MHC molecules, direct recognition is thought to occur because the allo- MHC molecule in association with a self-peptide resembles a self-MHC molecule plus foreign peptide at the three-dimensional level (Lechler *et al.*, 1990). In contrast, the indirect pathway involves recognition of specific fragments of foreign MHC molecules and all other proteins shed from the graft that are recognised as antigenic determinants bound in the groove of self-MHC molecules. This is the same mechanism of presentation as non-allorecognition. Experiments have shown that indirect allorecognition is involved in both acute and chronic allograft rejection in murine models (Fluck *et al.*, 1999). These elegant experiments investigated mouse cardiac transplantation in animals that were pre-conditioned to abrogate any direct allorecognition response from CD4+ cells. The CD4+ cells were restricted to recognising alloantigen only in the context of recipient MHC II. The study revealed that mice that were depleted of CD8+ cells alone rejected the grafts slowly with histological evidence of acute and chronic rejection, whereas those that were depleted of CD4+ cells alone accepted their grafts indefinitely albeit with evidence of heart damage. Those animals that were depleted of peripheral CD8+ cells alone were found to have CD8+ cells infiltrating the graft. This did not occur in the CD4+ and CD8+ depleted animals suggesting a role for CD4+ cells in recruitment of CD8+ cells into the graft.

There is some *in vitro* evidence suggesting a role for indirect allorecognition of MHC-allopeptides in human renal transplant recipients with chronic graft dysfunction (Vella *et al.*, 1997). Nevertheless, in a recent study of acute skin graft rejection greater than 90% of the anti-donor T cell repertoire was directed against

intact donor MHC molecules, while less than 10% of the response was directed against indirectly presented, donor derived peptides (Benichou *et al.*, 1999).

Rejection of MHC-identical grafts is triggered by products of genetic loci other than the MHC, termed minor histocompatibility antigens (reviewed by Goulmey, 1997). Evidence for the involvement of minor histocompatibility antigens (mHag) in human transplantation comes from data from bone marrow transplantation (Goulmey, 1997). Both cytotoxic and T-helper cells recognise mHag in a classical MHC fashion (Goulmy, *et al.*, 1976). The mHag are naturally processed fragments of intracellular proteins that associate with MHC molecules (Den Haan *et al.*, 1995). Well defined mHag include the male-specific mHag H-Y. The male specific antigen H-Y has been implicated in the rejection of MHC-matched bone marrow transplant recipients. For example, female patients that had been sensitised to H-Y following multiple blood transfusions developed bone marrow graft failure. *In vitro* analysis demonstrated the presence of HLA-A1-restricted anti- H-Y cytotoxic T-lymphocytes (Goulmy, 1997). The significance of H-Y in recipients that had not been previously sensitised is not known. Non-Y-linked mHag, for example HA-1, have been described in recipients of bone marrow transplantation and may play a role in graft failure of MHC-matched recipients (Goulmy, 1997). The role of mHag mismatches in renal transplantation is not clear although there was no effect of H-Y mHag in MHC-matched living-donor renal transplants (Ellison *et al.*, 1994).

1.2.2.3 Effecting the immune response to an allograft

Donor cells express MHC (HLA in humans) molecules on their surface. HLA class II antigens are recognised by CD4+ 'helper' T cells while HLA class I antigens are recognised by CD8+ cytotoxic T cells. Therefore mismatches between donor and recipient in HLA Class II antigens ('DR' region) will lead to activation of CD4+ cells. The host immune systems initial contact with the allograft is *via* leakage of 'passenger leucocytes' from the graft. These cells are donor haematopoietic cells that are passively transferred with the graft (Lechler *et al.*, 1982). After transplantation, graft-borne APC' leave the allograft and migrate to draining lymph nodes where they mature and acquire potent T cell activating properties (Larsen *et al.*, 1990). In the lymph node, passenger leucocytes provide donor MHC Class I and Class II molecules. Both CD4+ and CD8+ T cells, which recognise donor alloantigen via the direct pathway, become activated.

Once the T cells have completed their differentiation in the lymphoid tissue they home into the graft and find the appropriate target cells bearing their specific peptide/MHC complex. This process is facilitated by the secretion from the graft vascular endothelium of chemo-attractant molecules, called chemokines, and the upregulation of adhesion molecules on the surface of the graft vascular endothelium (Fuggle and Koo, 1998). Graft tissue is destroyed by specific cytotoxic T lymphocytes (CTL) and by a delayed hypersensitivity (DTH)-process in which activated macrophages and cytokines damage the graft. Alloreactive T cells, particularly a subcategory of CD4+ cells referred to as Th1 cells, activate

macrophages by the release of the cytokine γ-interferon and co-stimulatory signals. Once activated, macrophages release a variety of destructive mediators including nitric oxide, oxygen radicals and proteases.

CD8+ CTLs are able to destroy cells by two distinct mechanisms. Firstly, after binding the specific peptide/MHC Class I complex on to the graft cell, the CTL re-orientates its cytoskeleton to focus the release of lytic granules towards the target cell. CTLs may also utilize the Fas/Fas ligand pathway to induce apoptosis in the target cells (Strehlau *et al.*, 1997). These cell-mediated effects lead to the clinical picture of allograft rejection.

Allo-antibody responses can also contribute to graft rejection. Preformed antibodies directed against MHC or blood group antigens (ABO) can induce hyper acute rejection, a complement-dependent reaction in which endothelial destruction results in loss of the graft through ischaemia. Serological HLA crossmatch procedures identify donor-specific HLA or other alloantibodies in the recipient's sera that cause such early graft loss. A positive crossmatch would contra-indicate the transplant.

Rejection is therefore a complex process with a vast array of cellular and chemical interactions that ultimately lead to tissue and organ destruction and dysfunction that is seen in clinical transplantation. How can we extrapolate this basic knowledge to develop drugs that prevent this process occurring? It is clear from the above discussion that the primary target of immunosuppresive drugs is the T cell.

1.3 Effect of immunosuppressive drugs on the immune response

1.3.1 Introduction

Both Cy A and FK506 are calcineurin inhibitors sharing the same common pathway for their effects on the T-cell response to antigens (Siekierka *et al.*, 1989, Harding *et al.*, 1989, Johansson and Moller, 1990). Rapamycin and the analogue SDZ RAD act on T cell maturation events downstream from the initial T cell activation.

1.3.2 Mechanism of T cell activation following antigen recognition in relation to the Calcineurin Inhibitors

TCR engagement is referred to as Signal one. However, it has been established that the proximal events during T cell activation do not occur in the absence of a further signals known as Signal two. The best characterised 'co-stimulatory' pathway is that mediated by the CD28 surface receptor, which is present on 80% of human T cells (Linsley *et al.*, 1993). Two natural ligands of CD28 have been cloned, B7-1 (CD80) and B7-2 (CD86)). Both are expressed exclusively on activated APC. A second pathway in addition to CD28 that provides co-stimulation is mediated through the CD40 molecule on APC, which binds to CD40-ligand (CD40L) on activated T cells (Larsen and Pearson, 1997). A homologue of CD28, CTLA4 is expressed on activated T cells and transduces a negative signal to the cell interior and may be important in terminating an immune

response (Walunas *et al.*, 1996). Following antigen recognition, and with the appropriate co-stimulatory signals, the initial step of T cell activation, a sustained rise of intracellular calcium, can occur. The high level of calcium must be present for two hours for T cells to be committed to activation (Imboden and Weiss, 1987). Once the extra calcium reaches threshold level it binds to the ubiquitous calcium dependent regulatory protein calmodulin. Calcium-calmodulin complexes can activate several enzymes, including protein kinases (leading to addition of phosphate groups to a protein), and protein phosphatases (removal of phosphate groups) such as calcineurin. Calcineurin is a three component phosphatase made up of an A chain, which is a catalytic subunit, a B chain, which is a calcium binding subunit, and finally the calmodulin protein, which is a calcium binding regulatory subunit (Hubbard and Klee, 1989).

Calcineurin has a pivotal role in downstream events leading to the transcription of growth cytokines particularly interleukin-2 (IL-2). The transcription of IL-2 is a crucial step in T cell activation since following IL-2 expression further T cell activation becomes antigen independent (Crabtree, 1989). T cell activation not only involves IL-2 secretion but also expression of the IL-2 receptor on the surface of the cell (Hatekeyama *et al.*, 1989). The IL-2 receptor is upregulated by IL-2 and is a heterotrimeric recognition structure (Smith and Cantrell, 1984). The α chain (CD25), undergoes major changes in expression upon T-cell activation whereas the β- chain and the γ-chain are largely constitutively expressed on resting lymphocytes (Hatekeyama *et al.*, 1989; Takeshita *et al.*, 1992).

The intervening step between calcineurin activation and transcription of IL-2 is the translocation of NF-AT (Nuclear Factors of Activated T cells) from the cytoplasm to the nucleus. NFAT transcription factors are highly phosphorylated proteins (Crabtree, 1999). NFAT proteins are located within the cytoplasm of resting cells; upon activation they are dephosphorylated by calcineurin and transported into the nucleus. Mass spectrometry studies have shown that NFAT1 is phosphorylated on fourteen conserved phosphoserine residues in its regulatory domain, thirteen of which are dephosphorylated upon stimulation (Okamura *et al.*, 2000). Full dephosphorylation of the thirteen residues appears to be required to cause full exposure of an identified nuclear localization signal, mask a nuclear export sequence and allow maximal transcriptional activity (Okamura *et al.*, 2000). A summary of the events of T cell activation is represented in Figure 1.2.

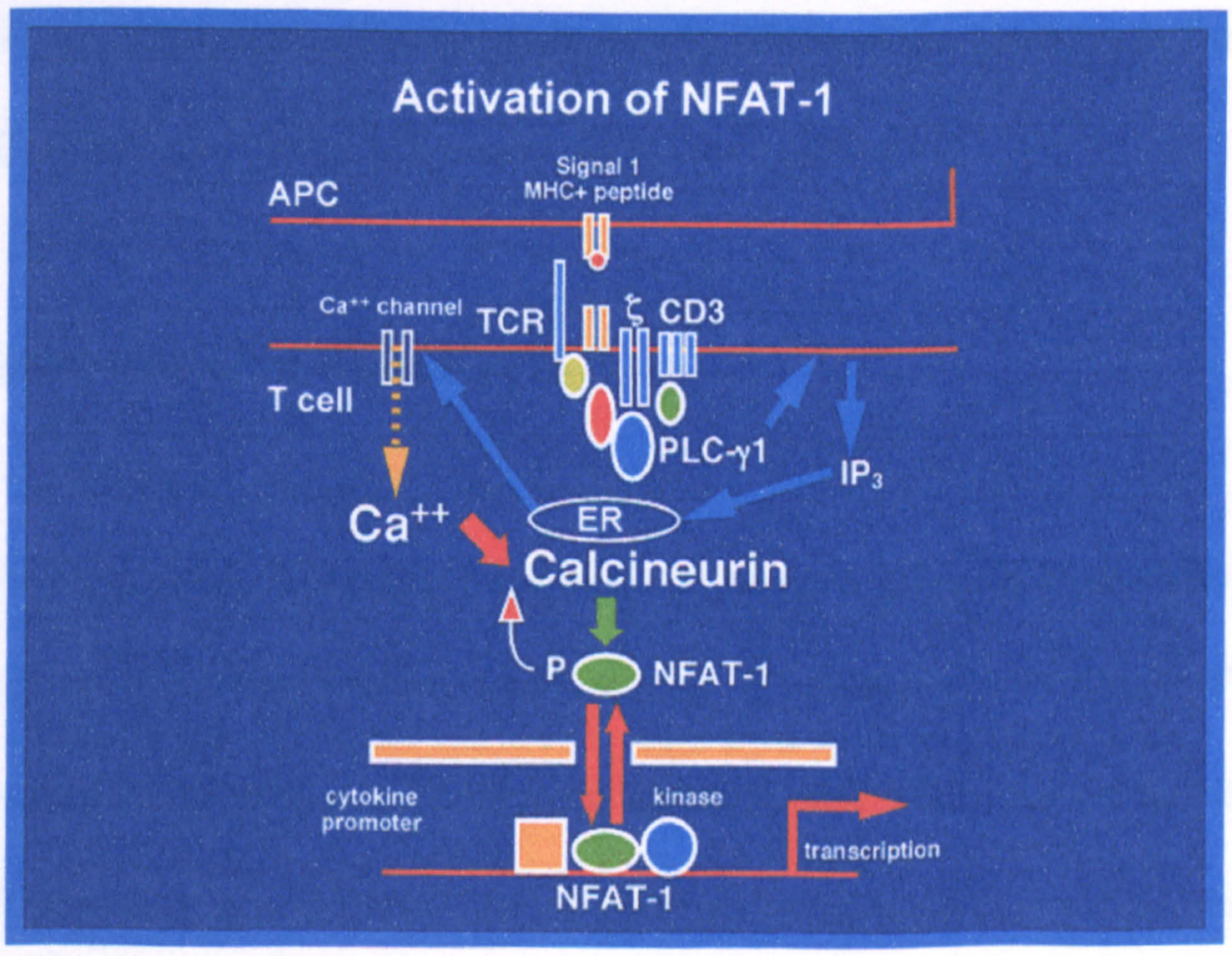


Figure 1.2 T cell activation. T cell receptor engagement by antigen in the form of MHC-peptide complex leads to the cascade of intracellular events and activation of the calcium-calmodulin dependent phosphatase, calcineurin. Calcineurin cleaves a phosphate moiety from NFATc, which allows the translocation of NFAT from the cytoplasm to the nucleus and the transcription of genes for growth cytokines.

1.3.3 Mechanism of action of Cy A

Cy A is a naturally occurring cyclic peptide isolated from a fungus, *Tolypocladium inflatams gams* It binds to many cellular targets, including the ubiquitous soluble cytosolic protein cyclophilin (Handschumacher *et al.*, 1984). Cyclophilin is identical to peptidyl-prolyl isomerase, an enzyme that catalyses the interconversion of the cis- and trans-rotamers of the peptidyl-prolyl amide bond of peptide and protein substrates (Fischer *et al.*, 1989). This rotamase activity is potently inhibited by Cy A (Siekierka *et al.*, 1989). Cy A-cyclophilin complexes can bind to calcineurin (CN), inhibiting the action of CN (Liu *et al.*, 1991). The dephosphorylation of NFAT does not occur in this situation and therefore NFAT remain in the cytoplasm (Timmerman *et al.*, 1996). This leads to failure of the early differentiation events associated with T-cell activation.

1.3.4 Mechanism of action of FK506

FK506 is a macrolide antibiotic isolated from *Streptomyces tsukubaensis* (Goto *et al.*, 1987). *In vitro* experiments confirm that in comparison to Cy A, FK506 is 40-200 times more potent at inhibiting mitogen stimulated responses and allo-mixed lymphocyte reactions of murine splenic lymphocytes in a dose dependent manner (Sawada *et al.*, 1987).

FK506 binds to a family of intracellular receptors termed, FKBPs or FK binding proteins (Fruman, Burakoff and Bierer, 1993). The principal cytoplasmic isoform

of the FKBPs is FKBP12, a 12-kD protein and like cyclophilin, FKBP has rotamase activity towards a peptide substrate (Siekierka *et al.*, 1989, Harding *et al.*, 1989). FKBP and cyclophilin are referred to as immunophilins. FK506 inhibits the rotamase activity of FKBP, but not of cyclophilin; likewise, Cy A does not inhibit the rotamase activity of FKBP (Siekierka *et al.*, 1989). The immunophilins are abundant cytoplasmic proteins and FKBP12 has been estimated to account for 0.3-0.5% of cellular proteins (Siekierka *et al.*, 1989). In addition, the immunophilin proteins are conserved throughout evolution; proteins with homology to these proteins have been found in all eukaryotic species as well as in E. coli and plants (Fruman, Burakoff and Bierer, 1994). This suggests that these proteins mediate essential cellular functions such as protein folding and cellular trafficking (Gething and Sambrook, 1992). As with Cy A/cyclophilin complexes the complex of FK506/FKBP inhibits the action of calcineurin (Liu *et al.*, 1991). Experiments utilising an FK506 derived radioactive ligand confirm that half maximal T cell suppression occurs following occupancy of FKBP12 of only 3-5% of the available pool (Dumont *et al.*, 1994).

1.3.5 Mechanism of action of Rapamycin

Rapamycin is a macrolide antibiotic product of *Streptomyces hygroscopius*, an actinomycete, originally isolated from a soil sample in Rapa Nui (Easter Island) in 1975 (Sehgal *et al.*, 1975). It has a similar structure to FK506 and binds to the same immunophilin as FK506 (Schreiber, 1991). However, the complex of rapamycin/FKBP is not able to inhibit the action of CN. Instead rapamycin is able to abrogate enzymes of the signal transduction cascade utilised by IL-2 to induce

T cell activation (Dumont *et al.*, 1990b). Moreover, FK506 and rapamycin can antagonise each other's immunosuppressive effects (Dumont *et al.*, 1990a; Bierer *et al.*, 1990), consistent with the idea that the drugs share the same intracellular binding sites.

The phosphorylation of 40S ribosomal protein S6 appears to be a prerequisite for the activation of protein synthesis and cell growth (Thomas *et al.*, 1982). This event is largely regulated by the mitogen-activated p70 S6 kinase in mammalian cells. The complex formed between FKBP12 and rapamycin abrogates the enzymatic activity of the p70 S6 kinase (Chung *et al.*, 1992) preventing cells from progressing from G1 to the S phase of the cell cycle. The precise target for the FKBP12-rapamycin complex has been identified as a novel protein named mammalian target of rapamycin (mTOR) by Sabers *et al* (1995) or FRAP (Brown *et al.*, 1994) or RAFT (Sabatini *et al.*, 1994). How mTOR directly affects p70 S6 kinase is not known.

Since rapamycin does not primarily affect early events after T cell activation, it is a less effective inhibitor of cytokine synthesis than Cy A or FK506. Unlike the CN inhibitors, rapamycin blocks calcium-independent pathways in T and B cells and also blocks activation pathways following stimulation with IL-2 and other growth cytokines (Kay *et al.*, 1991). Rapamycin blocks mitogenic stimulation of B cell proliferation (Wu *et al.*, 1991) and immunoglobulin synthesis. Although the anti-proliferative effects of Cy A and FK506 are primarily restricted to T and B cells, rapamycin can inhibit growth factor induced cell proliferation of fibroblasts,

endothelial cells, hepatocytes and smooth muscle cells (Akselband *et al.*, 1991 and Cao *et al.*, 1995).

The fact that rapamycin does not inhibit CN may account for the different toxicity profile compared to both Cy A and FK506. For example, rapamycin is non-nephrotoxic although does cause dose dependent lipid abnormalities with hypertriglyceridaemia and hypercholesterolaemia reported in approximately 50% of the patients and thrombocytopenia in 37% of patients (Groth *et al.*, 1999).

There are wide inter-individual variations in the pharmacokinetic properties observed upon oral administration in both animals and humans (Kahan *et al.*, 1995a). This has prompted the development of rapamycin analogues with more favourable pharmacokinetics, such as SDZ RAD.

1.3.6 SDZ RAD

SDZ RAD bears a stable 2-hydroxyethyl chain substitution at position 40 on the rapamycin structure and is a rapamycin analogue developed by Novartis (Basel, Switzerland). SDZ RAD suppresses T cell immune responses, by its ability to inhibit T cell proliferation stimulated by IL-2, as well as T-cell dependent and T-cell independent B cell responses. SDZ RAD binds with high affinity to FKBP12, although is about three-fold weaker than rapamycin (Schuler *et al.*, 1997). In general the *in vitro* activity of SDZ RAD is about two to three times lower than rapamycin and this level of effect is consistent with the lower affinity for FKBP12 (Schuurman *et al.*, 1997).

SDZ RAD has been shown to be effective in *in vitro* and *in vivo* models with dose dependent anti-proliferative effects on 2 way mouse mixed lymphocyte reaction (MLR) and prolongation of orthotopic kidney and cardiac allografting in the stringent DA-Lewis rat strain combination (Schuurman *et al.*, 1997 and 2000). Synergism with Cy A has been demonstrated (Schuurman *et al.*, 1997). Synergism is the enhanced effect of 2 compounds above that expected purely by addition of their individual effects. As Cy A and macrolides, such as rapamycin and SDZ RAD, differ in their respective side effect profile, this synergism may provide a similar level of efficacy with fewer side effects. For example, in the rat cardiac allograft model low dose SDZ RAD in combination with Cy A yielded long-term survival (Schuurman *et al.*, 1997). In the same model the maximum tolerated dose of each drug was less effective in terms of allograft survival. (Schuurman *et al.*, 1997).

1.3.7 Summary of the effects of immunosuppressive drugs on the immune response

Cyclosporin A and FK506 are potent inhibitors of T cell activation and the immune response to an allograft. Although they have different structures they mediate their actions through the phosphatase, calcineurin. They bind to different intracellular receptors called immunophilins. Both rapamycin and SDZ RAD are powerful immunosuppressant compounds. Binding to FKBP12 is central to their immunosuppressive action. However, unlike FK506, which also binds to FKBP12, they exert their effects on growth factor stimulated cell proliferation at the ribosomal level.

1.4. Pharmacokinetics of the calcineurin inhibitors in clinical transplantation

1.4.1 Introduction

The application of these powerful compounds in clinical practice is evolving continuously. The pharmacokinetics of the developed drugs has a significant effect on how they are utilised in clinical practice. The pharmacokinetics of Cy A and FK506 are discussed below.

1.4.2 Blood level monitoring of Cy A

Cy A is insoluble and this has made the preparation of reliable pharmaceutical formulations difficult. Conventional Cy A (or Sandimmun®) is an incomplete emulsification that produces wide variations in bioavailability (Kahan *et al.*, 1995b). Recently, the microemulsion formulation of Cy A, Neoral®, has been introduced into clinical use. Cy A has a narrow therapeutic window and in clinical transplantation it is important to monitor the blood level frequently (Calne *et al.*, 1983). The most widely accepted practice is to monitor the 'trough' blood level (C-0) just prior to the next dose. Accepted trough blood level ranges for Cy A are 100-400 ng/ml with higher levels for the induction phase of treatment during the first 3 months and subsequently lower maintenance levels. At these levels, Cy A inhibits about 50% of CN activity in *in vitro* experiments. Higher levels such as 800-1000ng/ml will lead to approximately 80% inhibition of CN but with increasing toxicity (Halloran *et al.*, 1999b). The pharmacokinetics of Cy A

administration have been extensively investigated. It has been demonstrated that conventional Cy A (Sandimmun(R)) trough blood levels do not predict clinical outcome in terms of acute rejection episodes and one year graft survival (Kahan *et al.*, 1996). However, the report also demonstrated that total drug exposure as measured by determining the Area Under the drug Concentration/Time curve (AUC) could be correlated with clinical outcomes. Despite this, it is not routine clinical practice to perform detailed AUC measurements since these are cumbersome and time consuming. The 'trough' blood level remains the most common vehicle for dose adjustment.

The new formulation, Neoral® is better absorbed and gives more consistent blood levels. However, as with 'conventional' Cy A (Sandimmun®), the trough blood levels do not give a truly accurate guide to total drug exposure, since there is a wide variation in absorption over the first 2-4 hours following dosing (Johnston *et al.*, 2000). This is important since most of the pharmacodynamic effects of Cy A occur within 2 hours (Halloran *et al.*, 1999b). Therefore, it can be envisaged that trough blood levels do not accurately represent drug exposure during the critical first few hours post dose. Trough blood levels following dosing with the Neoral(R) formulation do not reflect clinical outcomes in terms of acute rejection rates (Mahalati *et al.*, 1999). The data show that although high trough levels were associated with increased nephrotoxicity the trough levels did not predict those patients in whom acute rejection occurs. A drug level taken at 2 hours following ingestion correlates well with the formal AUC measurements and has been shown to be predictive of nephrotoxicity and acute rejection episodes (Mahalati *et al.*, 2000).

1.4.3 FK506 blood monitoring

Absorption of FK506 following oral administration is erratic with oral bioavailability varying from 5-67% (Venkataramanan *et al.*, 1991). There is marked inter-individual and intra-individual variation in the dose-normalised AUC following administration of FK506 in renal transplant recipients (Ihara *et al.*, 1995) However, there was good correlation between trough levels and the highest drug level reached, Cmax and $AUC_{0\text{-}12hours}$. Trough levels offer an acceptable practical way to monitor blood levels of FK506 (Ihara *et al.*, 1995). In kidney transplant patients the trough level of FK506 is well correlated with acute rejection episodes and nephrotoxicity (Kershner and Fitzsimmons, 1996).

1.5 Developing hypotheses

1.5.1 Introduction

Resistant allograft rejection under Cy A-based immunosuppression remains a considerable problem. As discussed in section 1.1.5.2, Jordan *et al* (1994 and 1997) reported two series of renal transplant recipients receiving Cy A as their primary therapy (Jordan *et al.*, 1994 and 1997). These patients were rejecting their grafts despite apparently adequate trough blood levels and were 'salvaged' by conversion to FK506 in 74% of cases. The reason for this 'salvaging' effect of conversion to FK506 is not wholly defined although one can speculate on possible contributory factors.

1.5.2 Explaining Cy A 'failure'

Could apparent 'Cy A failure' be attributed to the pharmacokinetics of Cy A and the lack of correlation of trough levels and total drug exposure? Conversely, does FK506 offer the advantage that trough blood levels more accurately reflect drug exposure than with Cy A in both of the formulations, Sandimmun(R) and Neoral(R)? FK506 has been shown to be more potent than Cy A in both *in vitro* models of allo reactivity and in animal models. Could the salvaging effect be as a result of increased potency of Cy A? Alternatively, are there additional pharmacodynamic effects that may lead to the development of resistance to either drug following treatment?

Following consideration of all these points a novel approach to the problem of drug failure would be to explore the possibility of selective resistance developing to either drug. The hypothesis presented here is that resistance to the drugs either develops in lymphocytes following prolonged exposure to the drugs or may pre-exist. The sensitivities of lymphocytes from renal transplant patients to the drugs have been examined.

1.5.3 Drug resistance

Briefly, these experiments confirm a wide range of lymphocyte sensitivities to both Cy A and FK506 and the development of resistance to the drug used for treatment. The potential cause of these observations has been investigated. The next part of the thesis (section 1.6) explores the concept of multidrug resistance.

1.5.4 Drug interactions

FK506 and rapamycin share the same intracellular binding sites and yet have different anti-proliferative targets within the cell (reviewed by Bierer, 1994). Studies on rapamycin confirm mutual antagonism with FK506 and led to the theory of a shared intracellular binding site (Dumont *et al.*, 1990a). However, a study in a rat cardiac transplantation model did not demonstrate antagonism but an enhanced immunosuppressant effect of combining rapamycin with FK506 (Vu *et al.*, 1997). Similarly, the combination of FK506 and rapamycin has been used as therapy for Experimental Autoimmune Uveoretinitis (EAU) (Ikeda *et al.*, 1997).

The combination therapy with FK506 and rapamycin achieved 100% inhibition of clinical EAU in a rat model, and 67% suppression of histological EAU. Therapy with either drug alone achieved only partial clinical suppression (Ikeda *et al.*, 1997).

These reports contradict the notion that the two drugs are mutual antagonists. However, likely explanations for these effects have been offered. Firstly, in Dumont's early report the two drugs, FK506 and rapamycin were mutually antagonistic when either drug was present in much higher quantities than the other (Dumont *et al.*, 1990a). Secondly, the mutual intracellular binding protein, FKBP12, is present within the cell in very high amounts, and this abundance *in vivo* could prevent competition (Siekierka *et al.*, 1989). Thirdly, only a small fraction of the cellular pool of FKBP12 needs to be occupied by the drugs in order to achieve high levels of immunosuppression (Dumont *et al.*, 1994). The investigation of the interaction of the two drugs, FK506 and the rapamycin analogue, SDZ RAD, in *in vitro* lymphocyte cultures from renal transplant patients is a worthwhile area of study. This is because using two drugs with predictable pharmacokinetic profiles in a synergistic way may lead to safer and more efficacious anti rejection protocols.

1.6. Multidrug Resistance

1.6.1 Introduction

Multidrug-resistance (MDR) is a phenomenon whereby cells that have been selected for resistance to one drug develop resistance to a wide variety of unrelated drugs (Endicott and Ling, 1989). The drugs most often involved in MDR are alkaloids, such as vincristine and vinblastine, or antibiotics of plant of fungal origin. MDR cell lines can be isolated *in vitro* by selecting cells for resistance to a single cytotoxic agent. The cells simultaneously become resistant to a number of other compounds. Although the drugs to which these cell lines are resistant appear to have no common structural or chemical properties, they tend to be large, planar, hydrophobic molecules (Endicott and Ling, 1989).

1.6.2 P-glycoprotein-170

One of the gene products mediating MDR was discovered in 1987 and is called P-glycoprotein-170 (P-gp). Transfection experiments using human P-gp cDNA sequences show that increased P-gp expression is able to confer a complete MDR phenotype on drug sensitive cells (Ueda *et al.*, 1987). The human P-gp genes map to the long arm of chromosome 7 at 7q21-31 (Trent and Witkowski, 1987). P-gp has been shown to be conserved across species. cDNA analysis has confirmed that there is a high degree of homology between different P-gp gene family members within a species and across species (Ng *et al.*, 1989). P-gp is now known to be the

170-kD product of the MDR1 gene, and is a member of the adenosine triphosphate [ATP]ase binding cassette (ABC) superfamily of active transporters (Hyde *et al*., 1990). P-gp is a transmembrane protein that is 1280 amino acids long and consists of two homologous halves of 610 amino acids joined by a flexible linker region of 60 amino acids. Each half has an N-terminal hydrophobic domain containing six transmembrane domains followed by a hydrophilic domain containing a nucleotide-binding site. The nucleotide-binding sites can bind ATP and its analogues and both sites are essential since inactivation of either site inhibits substrate-stimulated ATPase activity. The structure of P-gp has been determined to 2.5 nanometre (nm) resolution by electron microscopy and image analysis (Rosenberg *et al*., 1997). This is represented in Figure 1.3.

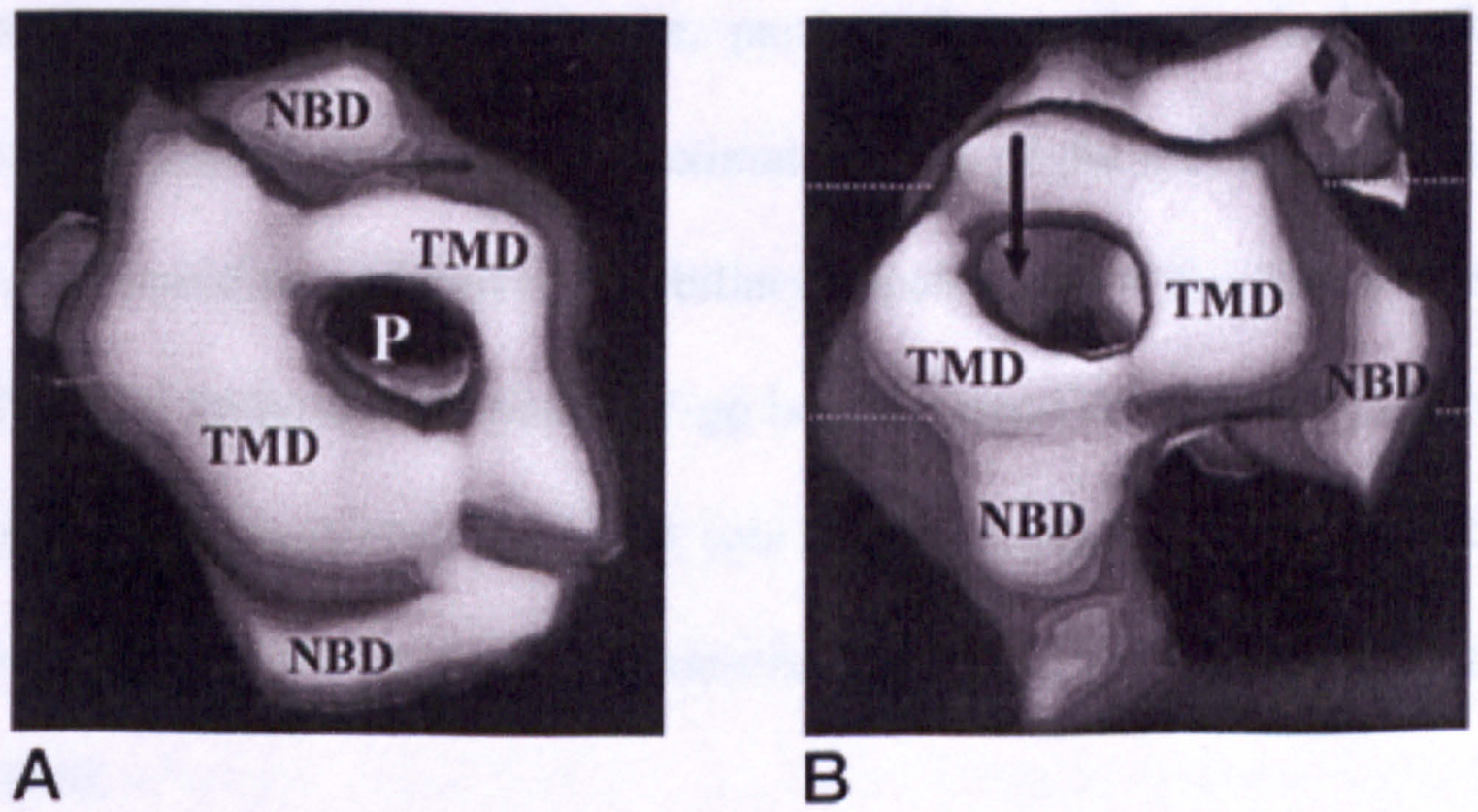


Figure 1.7 Three-dimensional structure of P-gp. 'P', aqueous pore open at the extracellular face of the membrane. ''TMD': transmembrane domains. 'NBD', Nucleotide-Binding Domains (annotated from Rosenberg *et al.,* 1997).

When viewed from above the plasma membrane, P-gp is donut shaped with 6-fold symmetry, a diameter of about 10nm and a large central pore of about 5nm in diameter. It has a thickness in the plane of the plasma membrane of about 8nm.

Since the depth of the plasma membrane lipid bilayer is about 4nm, about half of the molecule is within the plasma membrane. The central pore is closed at the inner (cytoplasmic) end, forming an aqueous chamber within the membrane that is open to the extracellular medium. There is an opening from this chamber to the lipid phase. Two lobes of about 3nm each are exposed at the cytoplasmic end and probably correspond to the nucleotide binding domains. The model proposed for this molecule is that it functions as an energy dependent transmembrane protein using ATP hydrolysis to pump cytotoxic drugs out of cells thus reducing their intracellular concentration and toxicity (Gottesman, 1993).

1.6.3 Physiological role for P-gp

P-gp is expressed in normal tissues, particularly on the luminal surfaces of transporting epithelia, such as the proximal tubules of the kidney, epithelial cells of the small intestine and colon, and biliary hepatocytes of the liver (Thiebaut *et al.*, 1987). The tissue distribution of P-gp is consistent with a membrane transport function. By homology to its transport role in MDR cells P-gp has been proposed to protect the organism from harmful natural toxins and xenobiotics (Endicott and Ling, 1989).

Monoclonal antibodies provide a method for determining the expression of P-gp in normal and tumour samples. A number of antibodies have been characterised that recognise cytoplasmic domains and external epitopes of P-gp such as MRK16 (Hamada and Tsuruo, 1986). Other tools that have been vital to the investigation of MDR phenotype have been the fluorescent dyes such as rhodamine-123

(R-123) (Neyfakh *et al.*, 1988). These dyes are actively transported from cells and indicate drug pumping. The ability of cells to efflux these dyes from the intracellular compartment to the surrounding medium is a reflection of MDR phenotype activity since it can be blocked by known chemical inhibitors of P-gp (Feller *et al.*, 1995, Pilarski *et al.*, 1995, Broxterman *et al.*, 1996, Petriz *et al.*, 1997).

1.6.4 P-gp expression on human peripheral blood lymphocytes

It has been demonstrated that some normal mouse spleen cells and human peripheral blood mononuclear cells could rapidly efflux R-123 and that such efflux was blocked by known chemical inhibitors of P-gp (Neyfakh, 1988). These observations have been developed further to quantify the differential activity of P-gp in normal blood lymphocyte subsets (Chaudhary *et al.*, 1992, Klimecki *et al.*, 1994, Pilarski *et al.*, 1995, and Ludescher *et al.*, 1998). For example, Chaudhary's group demonstrated that human peripheral blood lymphocytes express P-gp, and that P-gp expression correlates with the efflux of fluorescent dyes from cells. The efflux was suppressed by chemical inhibitors of P-gp and also by a P-gp specific monoclonal antibody (Chaudhary *et al.*, 1992). Double labeling of cells with specific antibodies to lymphocyte subsets demonstrated which lymphocyte subsets were able to efflux the P-gp specific fluorescent probes. The majority (80-90%) of the CD8 positive cells effluxed the dye and stained positive for he specific monoclonal antibody. Some 90-95% of NK cells (CD56+ cells) were positive. In contrast only 40-50% of CD4+ cells were positive for dye efflux and antibody staining. There was no P-gp activity demonstrated on CD14+ monocytes

(Chaudhary *et al.*, 1992). Approximately 42% of total adult peripheral blood mononuclear cells exported dye; 58% appeared to have no active efflux pump (Pilarski *et al.*, 1995). Similarly, 64% of B cells and 52% of T cells expressed surface P-gp as evaluated by staining with the monoclonal antibody, MRK16, which recognises an external epitope of P-gp. The proportion of positive staining cells was lowest among children less than 10 years of age, increased in adulthood, and decreased after 60 years of age. B cells from blood and spleen included a substantial proportion of cells with efficient dye efflux. In contrast, B cells from thymus and cord blood had only minimal dye efflux (Pilarski *et al.*, 1995). It was postulated that since there is developmentally regulated expression of P-gp function on lymphocytes that P-gp might have an important function in normal immune development. It has been shown that T cells with naïve phenotype have greater expression of P-gp than memory T cells (Ludescher *et al.*, 1998). The differential expression of P-gp activity suggests a physiological role for P-gp in lymphocyte function (Pilarski *et al.*, 1995). For example, P-gp has a putative role as a transporter of cytokines (Drach *et al.*, 1996). Upon stimulation, lymphocytes exert their immunological actions in part *via* production of cytokines. The release of IL-2, IL-4 and interferon-γ from stimulated normal lymphocytes is significantly suppressed in the presence of P-gp inhibitors although there was not significant down regulation of IL-2 mRNA expression (Drach *et al.*, 1996). Similarly, it has been demonstrated in mitogen stimulated lymphocyte cultures treated with monoclonal antibodies to P-gp have a significant reduction in IL-2 levels in the culture supernatant (Raghu *et al.*, 1996).

1.6.5 P-gp expression on tumours

Expression of the MDR1 gene in different types of solid tumours and leukaemias has been associated with increased clinical resistance to chemotherapy (Goldstein *et al.*, 1989). Considerable heterogeneity is seen in P-gp expression in human cancers. Retrospective studies on tumour samples taken sequentially from patients with neuroblastoma, childhood soft tissue sarcoma and retinoblastoma have demonstrated that P-gp expression inversely correlates with tumour responsiveness to chemotherapy (Chan *et al.*, 1990 and 1991).

1.6.6 Cyclosporin A and FK506 are substrates for P-gp

The role of MDR mechanisms in transplantation is unknown. However, it has been considered a worthwhile area of study since the report that human P-gp transports Cy A and FK506 (Saeki *et al.*, 1993). LLC-PK_1 cells derived from porcine kidney tubular cells were planted on bottom-filtered cups. Further cells were transfected with human MDR1 cDNA and a clone established after selection in colchicine. Tritiated Cy A (^{3}H-Cy A) was added to the medium of the basal side or of the apical side of the monolayer of cells. The transfected cells, termed LLC-GA5-COL300, express P-gp specifically on the apical surface. The amount of Cy A that passed across the transfected cells' epithelium from the basal to the apical surface reached 10% in 3 hours, which was higher than the 4.5% which passed through the non transfected cells. The corresponding amount that traversed from apical to the basal side was only 3.9%. When the ^{3}H-Cy A content of the

cells was measured it was found that only one sixth of the amount was present in the transfected cells compared to the non-transfected cells when ^{3}H-Cy A was added to the apical side. The experiments were repeated with FK506. The amounts of FK506 that passed from the basal to the apical side of the LLC-PK1 epithelium were 27 and 26% respectively, indicating passage by simple diffusion. The amount passed across the transfected cell monolayer from the basal to the apical side was 40%, and the amount from the apical to the basal side was only 10%, representing the typical pattern of oriented transport by P-gp. Accumulated FK506 in the transfected cells decreased to one fourth of that in the non-transfected cells. The conclusions from these experiments were that Cy A and FK506 are substrates for P-gp mediated transport (Saeki *et al.*, 1993).

1.6.7 The biological consequences of P-gp mediated Cy A and FK506 transport

There are both pharmacokinetic and pharmacodynamic consequences of P-gp mediated Cy A and FK506 transport. Cy A and FK506 are metabolised primarily by cytochrome P450 3A (CYP3A4) in the liver (Kronbach *et al.*, 1988 and Sattler *et al.*, 1992). However, small bowel epithelial cells express abundant quantities of CYP3A4 (Kolars *et al.*, 1992), and metabolism via this pathway accounts for 50% of oral Cy A metabolism (Kolars *et al.*, 1991). The quantity of intestinal P-gp accounts for approximately 17% of the variability in oral Cy A pharmacokinetics in a study of renal transplant patients (Lown *et al.*, 1997).

The pharmacodynamic effects of P-gp mediated transport of Cy A have been investigated (Fakata *et al.*, 1998). The hypothesis that increased expression of P-gp confers protection against Cy A dependent inhibition of CN phosphatase activity was explored on selected cell lines expressing varying levels of P-gp (Fakata *et al.*, 1998). The study group utilised the human epidermal carcinoma cell line KB-V that were grown in media supplemented with varying strengths of vinblastine. Dose response curves were then obtained for CN phosphatase activity against Cy A concentration. KB-V cells grown in high dose vinblastine shifted the dose response curve to the right some 70-fold compared to the parent KB cells. Verapamil, a P-gp inhibitor, completely extinguished the protection afforded by P-gp expression. There are no reports of similar experiments in the target cells for Cy A, namely, lymphocytes, in renal transplant patients. However, modulating the effects of P-gp on lymphocytes has been reported in respect to sensitivity to doxorubicin in the mouse (Pourtier-Manzanedo *et al.*, 1993). Concanavalin A-stimulated mouse spleen cells display a doxorubicin-resistant component. However, by adding inhibitors of P-gp, in this case the Cy A analogue SDZ 833, the T cell proliferative responses display a much higher doxorubicin sensitivity.

1.6.8 Non P-gp mediated MDR: Role of MRP and LRP

1.6.8.1 Introduction

P-gp, the phenotype of the MDR1 gene in man does not account for all MDR that has been observed to develop in the treatment of cancer. Studies have confirmed that drug selected cell lines have MDR activity but do not contain P-gp (Taylor *et*

al., 1991). One of the most extensively characterised non-P-gp multidrug-resistant cell line is H69AR, a small cell lung cancer cell line which was derived from the drug sensitive parental cell line H69 by step-wise selection in doxorubicin (Mirski *et al.*, 1987). The mRNA species that was over expressed in the resistant cells was identified and sequencing of cDNA clones derived from this mRNA revealed that it had the potential to encode a 1531 amino acid protein which was named multidrug resistance-associated protein (MRP). This protein is a member of the same ATP-dependent transmembrane transporter superfamily as P-gp (Cole *et al.*, 1992). Transfection of MRP cDNA confers a similar pattern of resistance to important cytotoxic drugs such as anthracyclines, Vinca alkaloids, and antibiotics (Cole *et al.*, 1992).

1.6.8.2 Physiological role for MRP

The physiological relevance of MRP is not established although MRP has been demonstrated to transport cysteinyl leukotriene, LTC4, and other glutathione conjugates (Zaman *et al.*, 1995). MRP mRNA is expressed at low levels in many tissues, including haematopoietic cells. Analysis of the tissue distribution of mRNAs for human and murine MRP indicate that the highest levels of expression are in the testes, skeletal muscle, heart, kidney and lung. Low levels are seen in liver and intestine (Kruh *et al.*, 1995). Reduced drug accumulation and enhanced drug efflux is usually observed in drug selected cells that over-express MRP (Zaman *et al.*, 1994). The expression of MRP in normal cells from peripheral blood and bone marrow has been examined (Legrand *et al.*, 1996). The MRP mRNA levels were estimated by reverse transcriptase/polymerase chain reaction

(RT/PCR) and *in situ* hybridization (ISH) assay. MRP was present in peripheral blood and bone marrow cells from normal donors, and those in remission after chemotherapy for large cell lymphoma or acute myeloid leukaemia. Individual expression levels were variable; however, there was no difference between untreated normal bone marrow and post chemotherapy bone marrow. MRP protein expression was determined by flow cytometry with the monoclonal antibody MRPm6. The CD4+ lymphocytes exhibited a higher MRP protein expression than the other cell lineages, including CD8+ cells (Legrand *et al.*, 1996).

1.6.8.3 Investigating the clinical importance of MRP

The role of MRP in chemotherapy failure remains poorly defined, although circumstantial evidence for a role has been reported. Firstly, deletion of the MRP gene on the inverted chromosome 16 of myelomonocytic leukaemia correlated with a better outcome (Kuss *et al.*, 1994). Secondly, MRP expression in retinoblastoma correlated with failure of chemotherapy despite inhibiting P-gp with Cy A (Chan *et al.*, 1997).

To facilitate studies of MRP several specific monoclonal antibodies have been developed which may lead to detection and characterisation in clinical tumour samples (Flens *et al.*, 1994 and Hipfner *et al.*, 1996). The majority of clinical data relating to MRP has been obtained in leukaemic cells. The studies of MRP in clinical specimens are difficult to interpret since the methods used to characterise the expression and functional activity of MRP are not standardized (Feller *et al.*,

1995). In a study in 65 heterogeneous leukaemic patients only 6% of cases expressed MRP in contrast to 53% expressing P-gp either alone or in combination with MRP or Lung Resistance Protein (LRP) (Michieli *et al.*, 1997).

1.6.8.4 Lung Resistance Protein

Lung-Resistance Protein has been described in non P-gp MDR. The LRP is the human major vault protein (Scheffer *et al.*, 1995). It is a 110-kD protein that has been associated with a poor prognosis in acute myeloid leukaemia and ovarian carcinoma when over expressed (Izquierdo *et al.*, 1995). It is expressed in all P-gp negative MDR cell lines tested so far. The LRP gene has been localised to the short arm of C-16 and this region appears to be critically involved in MDR (Scheffer *et al.*, 1995). A monoclonal antibody, LRP-56, has been used for the specific detection of LRP in frozen sections by immunohistochemistry and in flow cytometry (Izquierdo *et al.*, 1995 and Michieli *et al.*, 1997). In flow cytometry studies the parental cell lines and normal leucocytes reacted weakly to all the monoclonal antibodies tested and functional assays of LRP have not been established (Michieli *et al.*, 1997). Although LRP does have prognostic implications in certain malignancies as an important mediator of drug resistance, the lack of detection in normal peripheral blood mononuclear cells (PBMC) made the study of this and indeed the study of MRP in PBMC from renal transplant patients unattractive.

1.6.9 MDR inhibitors

1.6.9.1 Introduction

A characteristic of MDR is that it can be reversed by a number of diverse compounds which can act as chemosensitisers. These agents are not toxic to MDR cells at concentrations that reverse the MDR phenotype. When administered in combination with anti-cancer drugs they enhance cellular anti-cancer drug accumulation by acting as competitive inhibitors for drug transport. A number of compounds have been investigated for their ability to reverse the MDR phenotype. Examples include verapamil, amiodarone, phenothiazines, neuroleptics, and Cy A. However, many of these compounds produced severe toxic effects such as blocking ion channels or modulation of other physiological pathways (Endicott and Ling, 1989).

1.6.9.2 PSC 833

PSC 833 is a cyclosporin analogue that has been extensively studied with respect to inhibiting MDR (Twentyman and Bleehen, 1991, Boesch *et al.*, 1991 and Broxterman *et al.*, 1996). Resistant cell lines have been utilised to determine the potency and mechanism of action of PSC 833. For example, the MDR human breast adenocarcinoma cell lines were developed that express increasing levels of P-gp. At doses of 1μmolar, PSC 833 restored the sensitivity of these resistant cells to anticancer drugs (Atadja *et al.*, 1998). An investigation into the nature of the

interaction of PSC 833 with P-gp has been performed (Archinal-Mattheis *et al.*, 1995). Plasma membrane-enriched fractions from drug sensitive and resistant cells were labelled with photoreactive ^{3}H-cyclosporin and ^{3}H-PSC 833 derivatives. P-gp was detected only in membranes derived from drug-resistant cells. This photoaffinity labelling was competitively inhibited by excess PSC 833 or Cy A, demonstrating P-gp as the target molecule of PSC 833 and Cy A. Efflux experiments reveal no efflux of PSC 833 in drug sensitive or resistant cell lines, probably due to high affinity for P-gp (Archinal-Mattheis *et al.*, 1995). Further evidence for P-gp as the target of PSC833 comes from experiments on single point mutations in P-gp molecules. These mutant proteins when transfected into cell lines alter the MDR inhibiting characteristics of Cy A and PSC 833 (Watanabe *et al.*, 1996).

PSC 833 is being developed as a resistance modifier in cancer chemotherapy protocols. For, example, PSC 833 (Valspodar®) has completed phase I/II clinical trials (Boote *et al.*, 1996) in combination with etoposide for treatment of lung cancer and in combination with daunorubicin in acute myeloid leukaemia (Tidefelt *et al.*, 2000).

1.6.10 Clinical significance of MDR

There are no prospective data examining MDR mechanisms and outcome. The majority of the clinical studies have involved the treatment of leukaemia. There were demonstrable increased levels of MDR phenotype expression in relapsed and refractory cases of acute myeloid and lymphoblastic leukaemias (AML and ALL)

(Takeshita *et al.*, 1995). However, the clinical relevance in childhood acute lymphoblastic leukaemia is unknown (Den Boer *et al.*, 1997). A recent review of the published data for AML concluded that the effect of P-gp on outcome was weak (Holmes and West, 1994). Therefore, the relevance of the function of P-gp in clinical MDR will require multicentre prospective clinical trials (Broxterman *et al.*, 1996).

1.7 Final hypothesis

1.7.1 Explaining Cy A 'failure'

As discussed in section 1.5 "Developing hypothesis" the observation of Cy A failure and the subsequent salvaging of renal allografts with another calcineurin inhibitor, namely, FK506 was unexplained. Following consideration of all the pharmacokinetic and pharmacodynamic points that have illustrated during this introduction a novel approach to the problem of drug failure is an exploration of the possibility of selective resistance developing to either drug. The hypothesis is that resistance to the drugs either developed in lymphocytes following prolonged exposure to the drugs or may pre-exist.

1.7.2 Drug resistance

The sensitivities to Cy A and FK506 of lymphocytes from renal transplant patients were examined. Briefly, these experiments confirm a wide range of lymphocyte sensitivities to both Cy A and FK506 and the development of resistance to the drug used for treatment. The product of the MDR1 gene, P-gp may explain this variation in sensitivity and development of selective resistance to the drugs.

1.7.3 Drug interactions

FK506 and SDZ RAD share the same intracellular binding sites and yet have different anti-proliferative targets within the cell (see section 1.3). Studies on rapamycin confirmed mutual antagonism with FK506 (Dumont *et al.*, 1990). However, studies in animal models did not demonstrate antagonism but an enhanced immunosuppressant effect of combining rapamycin with FK506 (Vu *et al.*, 1997). The interaction of FK506 and the rapamycin analogue SDZ RAD in *in vitro* lymphocyte cultures from renal transplant patients was examined. This is a worthwhile area of study since the potential of using two drugs with predictable pharmacokinetic profiles in a synergistic way may lead to safer and more efficacious anti rejection protocols.

CHAPTER 2

AIMS AND OBJECTIVES

2 Aims and Objectives

The primary aim of the work described in this thesis was to assess the pharmacodynamic effects of immunosuppressive drugs on *in vitro* cultures of peripheral blood mononuclear cells (PBMC) from renal transplant patients. This work can be divided into the following areas of study:

1. An exploration of the *in vitro* methods of PBMC stimulation to test for variations in sensitivity of PBMC to immunosuppressive drugs.

2. An examination of the development of resistance to these drugs since this may predict individuals 'failing' on a particular drug.

3. The study of Multidrug-Resistance (MDR) mechanisms and an exploration of the relevance of MDR in PBMC from renal transplant patients

4. Finally, the investigation of the interactions between FK506 and the structurally related novel compound, SDZ RAD.

CHAPTER 3

INTRODUCTION TO EXPERIMENTAL METHODS

3.1 In vitro lymphocyte cultures

3.1.1 Introduction

To assess the pharmacodynamic effects of immunosuppressive drugs on peripheral blood mononuclear cells (PBMC') from renal transplant patients the cells must first be cultured and then stimulated to proliferate. Stimulation of the cultured cells can occur in the presence of mitogen or from taking advantage of alloreactivity by co-culturing cells from two different individuals, as in the mixed lymphocyte reaction.

3.1.2 Mitogen stimulation of PBMC

Concanavalin A, a plant lectin, is a T cell mitogen that has been used to stimulate T cells in *in vitro* cultures for many years (Kay and Benzie, 1983; Kay *et al.*, 1989 and Liu *et al.*, 1992). Additionally, plant lectins such as Con A and Phytohaemagglutinin (PHA) have been shown to stimulate cells in a way that is sensitive to the effects Cy A, FK506 and rapamycin (Kay *et al.*, 1989, 1991; Wu *et al.*, 1991 and Liu *et al.*, 1992). The Con A response shows a greater sensitivity to inhibition by Cy A than the response to PHA (Kay and Benzie, 1983). The mechanism of action of the plant lectin mitogens is unclear. They bind to a number of T cell membrane glycoproteins, and it has been suggested that PHA binds CD2, the 20 kD molecule of the CD3 complex and TCR (Valentine *et al.*, 1985). The responses obtained to Con A and PHA are dependent on the elevation

of the cytoplasmic calcium concentration. (Kay and Benzie, 1983 and Kay *et al.*, 1989). Polyclonal T cell activation can also be delivered using monoclonal antibodies directed at the CD3/TCR in the presence of accessory cells (Vine *et al.*, 1988). The T cell response in these systems is dramatically enhanced by including antibodies against the co-stimulatory receptor, CD28 (Linsley and Ledbetter, 1993). Similarly, an enhanced response to PHA has been demonstrated in T cells co-cultured with the anti-CD28 monoclonal antibody (Gmunder et al, 1984). In summary, lectin mitogens and anti-CD3 monoclonal antibodies are polyclonal stimulators of T cells in the presence of accessory cells. The use of Con A stimulation of mononuclear cells in *in vitro* cultures is a valuable tool to investigate the anti-proliferative effects of Cy A, FK506 and rapamycin.

3.1.3. Mixed lymphocyte reactions

The mixed lymphocyte reaction (MLR) is an? *in vitro* correlate for rejection responses (Hayry and Defendi, 1970). In the MLR, allogeneic stimulation of T cells leads to cytokine production and proliferation. This response is thought to mimic events occurring during transplant rejection. The MLR is induced by culturing mononuclear leucocytes (which include T cells, B cells, natural killer cells, mononuclear phagocytes, and dendritic cells) from one individual with cells derived from another individual. If there are differences in the alleles of the major histocompatibility complex (MHC) genes between the two individuals, a proportion of the cells, approximately 10%, will proliferate over a period of 4-7 days (Hayry and Defendi, 1970). This proliferative response can be measured by incorporation of tritiated (^{3}H)-thymidine into DNA during cell replication. To

simplify the analysis, one of the mononuclear cell populations can be rendered incapable of proliferation, either by gamma irradiation or by pre-treatment with mitomycin C, an anti-mitotic drug. This sets up a 'one way MLR'. The treated cells serve as stimulators and the responder cells proliferate. In general, the MLR depends on differences in the Class II MHC antigens between the responder and stimulator, although discrepancies at only Class I MHC antigens can stimulate a response. T cells do not express class II MHC antigens and therefore responses are seen in the MLR when T cells from the responder interact with B cells and monocytes from the stimulator population.

Despite the convenience and reproducibility of mitogen stimulated assays the MLR is the more specific allo-response. The MLR was therefore utilised to investigate and characterise immunosuppressive drug interactions. For these studies, surrogate donor cells were used as stimulators with the renal transplant recipients' cells being the responding cells. The surrogate donor cells were matched for the Class II MHC antigens present on the cells of the original kidney donor (see Table 4.2). The original donor cells were not available to use for these experiments.

3.1.4 Previous studies of variation of sensitivity to immunosuppressive drugs in *in vitro* cultures of PBMC

The pharmacodynamic assessment of individuals' lymphocyte responses to immunosuppressive drugs could be an efficient strategy for tailoring treatment protocols. Previous reports have attempted to predict clinical response to anti-rejection drugs based on an *in vitro* test of lymphocyte sensitivity.

(a) Francis (1988) studied MLR in preoperative samples from potential renal transplant patients and assessed sensitivities to anti-rejection drugs. The resistant group had a higher rate of graft loss from acute rejection, more episodes of graft rejection and higher levels of serum creatinine (Francis *et al.*, 1988).

(b) Transplant recipient lymphocyte sensitivity to methylprednisolone strongly influenced the survival of MLC-mismatched kidneys (Langhoff *et al.*, 1986).

(c) Hirano, 1997 performed drug sensitivity tests with PBMC from four subject groups: 69 healthy subjects, 100 patients with chronic renal failure of which 40 had received a renal transplant, 38 patients with nephrosis, and 51 patients with psoriasis (Hirano *et al.*, 1997). The values for the concentration of Cy A that produced 50% inhibition of lymphocyte proliferation (ID_{50}s) were estimated. The median ID_{50} values in the subject groups were broadly the same. However, there were large individual variations in ID_{50} values. A higher incidence of

resistance to Cy A was observed in the chronic renal failure group. The incidence of graft loss in those 5 patients in the low sensitivity group compared to the highly sensitive group (9 patients) was significantly higher and there was an association with higher serum creatinine in the low sensitivity group.

(d) Ghobrial (1994) performed a longitudinal study carried out on 19 recipients of cadaveric renal allografts, monitoring their anti-donor and anti-third party responses in the MLR. The study concluded that in some renal allograft recipients a state of donor-specific hyporesponsiveness develops, and this state was associated with better graft outcome at 1 year (Ghobrial *et al.*, 1994).

These studies attempted to predict clinical response to antirejection drugs based on an *in vitro* test of lymphocyte sensitivity. However, during the current project this effect was not assessed, simply, the *in vitro* methods of lymphocyte stimulation were utilised to test for variation in lymphocyte sensitivity to immunosuppressive drugs; the development of resistance to these drugs, and finally to explore the potential drug interactions between FK506 and SDZ RAD.

3.1.5 CD25 expression assays

Cell proliferation can be measured in different ways. Firstly, by incorporation of ^{3}H-thymidine into DNA during cell replication and secondly by incorporation of ^{35}methionine. Flow cytometric analysis of T cell activation markers such as CD25, the α-chain of the interleukin-2 receptor (IL-2R) can be used as surrogate markers of proliferation. The high affinity IL-2R consists of three individual polypeptides,

the α, β and γ chains. Although the β- and γ- chains are shared by other lymphokine receptors, the α-chain (also called Tac, or p55 protein) is restricted to the IL-2R and is strongly induced at the transcriptional level after T cell activation (Hatekeyama *et al.*, 1989). CD25 does not transduce a signal but is responsible for the rapid association of IL-2 with the β and γ chains, which in turn trigger the antigen activated T cell to enter mitosis and undergo clonal expansion (Robb *et al.*, 1984). Following the development of specific antibodies to CD25 it is possible to measure CD25 expression by flow cytometry. Mitogenic activation of T cells leads to upregulation of CD25 expression that is sensitive to the effects of the immunosuppressant drugs Cy A, FK506 and rapamycin (Ryffel *et al.*, 1995 and Woerly *et al.*, 1996). This effect is dependent on the type of mitogen chosen. T cell activation by anti-CD3-antibody, for example, that utilises early calcium dependent pathways in a similar way to Con A and PHA, is fully sensitive to inhibition by the immunosuppressants mentioned above. Conversely, PMA-induced CD25 expression is drug resistant (Ryffel *et al.*, 1995).

3.1.6 Conclusion: *In vitro* assays of lymphocyte sensitivity

Con A stimulation of PBMC is an established and reproducible method for investigation of lymphocyte sensitivity to immunosuppressive drugs, the investigation of the development of selective resistance to these drugs and for assessment of interactions between them. The MLR offers a more physiological stimulus, however, the low level of cell stimulation in MHC Class II matched donor/recipient pairs renders the investigation of drug effects on the cells difficult.

3.2 Investigating Multidrug Resistance

3.2.1 Introduction

To be able to characterise expression and functional activity of MDR in clinical specimens requires specific probes. Investigators have utilised both monoclonal antibodies (Mab) and drugs that accumulate in cells unless actively pumped out of by MDR phenotypes (Hamada and Tsuruo, 1986, Pilarski *et al.*, 1995, Broxterman *et al.*, 1997). Finally, the effects of MDR modifiers have been explored in an effort to develop a system that is specific for the MDR phenotype of interest (Feller *et al.*, 1995, Pilarski *et al.*, 1995, Broxterman *et al.*, 1996, Petriz *et al.*, 1997). There is not a universally agreed specific system employed by investigators. The experimental systems continue to evolve and are modified to improve specificity and sensitivity. During international workshops discussions continue about standardisation of MDR detection in clinical samples (Beck *et al.*, 1996).

3.2.2 Flow Cytometry

The flow cytometer is a versatile tool for the investigation of individual cells within larger populations. For every cell, numerous light scattering and fluorescent properties, each reflecting an underlying structural, biochemical and genetic attribute of the cell, can be measured simultaneously. The method requires as its raw material a suspension of single cells or nuclei, which is usually obtained

from fresh disaggregated biological tissues or cultured cells. During the current thesis monoclonal antibodies to specific antigens have been used to identify lymphocyte subsets and explore P-gp expression. After the primary antibody is attached to the antigen in question the next step is to label the antibody with a microscopically dense marker in order to make it visible. There are several ways of doing this but because of the reliability and ease of performance we used fluorescein isothiocyanate (FITC-) or phycoerythrin (PE-) labelled conjugates. In essence, FITC- and PE- are fluorescent probes that can be conjugated directly to the primary antibody or conjugated to a secondary antibody raised against the primary antibody. Non-immunological attachment of antibodies and dense markers to the cells can result in non-specific staining. Blocking reagents such as bovine serum albumin or goats serum contain proteins that compete with the immunological reagents for non-immunological sticky sites on the cells. In order to allow for non-specific binding isotype matched control antibodies are used. The isotype refers to the immunoglobulin subclass that the antibody contains. The flow cytometer is set to discriminate lymphocytes from other nucleated cells by their light scatter characteristics. The cells are defined in a two dimensional plot by forward scatter of light and side scatter. Forward Scatter (cell size) versus Side Scatter (cell granularity) identifies the cell population of interest. The FITC- or PE- light emissions are collected from 5000-10000 lymphocytes and stored in list mode data files prior to analysis. The flow cytometer can also detect cellular accumulation of fluorescent dyes such as rhodamine 123.

3.2.3 Detection of P-glycoprotein 170 expression

Flow cytometry has been mainly used for the detection of surface membrane antigens such as those P-glycoprotein-170 (P-gp) epitopes recognised by monoclonal antibodies, MRK16, 4E3 and JSB-1 (Pilarski *et al.*, 1995, Michieli *et al.*, 1997, Klimecki *et al.*, 1994, Broxterman *et al.*, 1996, Delville *et al.*, 1995). The advantage of these antibodies is that they can stain viable cells with intact cell membranes. There is variation in reporting of P-gp expression on PBMC from normal volunteers and patients. There have been two reports of high levels of expression of P-gp following binding with the MRK16 monoclonal antibody allowing characterisation of lymphocyte subset expression (Klimecki *et al.*, 1994 and Pilarski *et al.*, 1995). A further study showed P-gp functional activity but no detectable P-gp expression with a further characterised monoclonal antibody to P-gp, JSB-1 (Delville *et al.*, 1995). Following the recommendations in the literature as outlined above the monoclonal antibodies to external epitopes of P-gp 4E3 and MRK16 were used. The MRK16 monoclonal antibody is a mouse anti-human P-gp. The antibody reacts with P-gp exposed on the cell surface, which allows labelling of living cells (Hamada and Tsuruo, 1986). Similarly, 4E3 is a mouse monoclonal antibody of IgG2a class, which specifically recognises the human MDR1 gene product P-gp. 4E3 is generally less reliable than MRK16 and is more batch dependent (Den Boer *et al.*, 1997).

The human breast carcinoma cell line MCF-7 (originally described by Soule *et al.*, 1973) is rendered resistant to chemotherapeutic agents by exposure to, for

example, doxorubicin or adriamycin (ADR) in increasing concentrations. Such selected cell lines have been used frequently as models for the study of MDR (Davies *et al.*, 1996) and this cell line served as a positive control for P-gp expression and functional activity.

3.2.4 Functional assays of MDR

3.2.4.1 Rhodamine 123

The development of fluorescent dyes that behave like chemotherapeutic drugs without any deleterious effects on cell biology or viability serve as valuable alternatives to functional studies with the drug itself (Neyfakh, 1988). Rhodamine 123 (R-123) is a cationic, lipophilic fluorochrome, which accumulates rapidly in cells, preferentially in the mitochondria membranes of living cells (Johnson *et al.*, 1980). Cell viability following exposure to R-123 remains very high and has been shown to be around 95% (Delville *et al.*, 1995). R-123 is transported out of cells by P-gp (Neyfakh *et al.*, 1988). Many studies have utilised R-123 retention and expulsion from cells to test for MDR and by performing combined experiments with specific P-gp Mab have concluded that R-123 expulsion is secondary to P-gp efflux mechanisms. In addition the use of P-gp inhibitors have demonstrated that R-123 efflux can be specifically inhibited or modulated (Chaudhary *et al.*, 1992, Ludescher *et al.*, 1992 and 1998, Broxterman *et al.*, 1996, Feller *et al.*, 1995, Pilarski *et al.*, 1995, Petriz *et al*, 1997). P-gp -mediated transport of R-123 has also been established by demonstrating that it is efficiently effluxed by a P-gp-expressing subline of K562 leukaemia cells (derived from retroviral transfer of

MDR1 gene, without cytotoxic selection) but not by the parental P-gp negative K562 (Chaudhary and Rominson, 1991). R-123 accumulation is an insensitive way to measure the transport function of non-P-gp MDR in the form of MRP, inferring selectivity for P-gp (Feller *et al.*, 1995). Other probes tested for P-gp functional activity include the anthracycline, daunorubicin (DNR) and calcein-acetoxymethyl ester (calcein-AM). However, both DNR and Calcein-AM could also be used to detect MRP-mediated resistance (Feller *et al.*, 1995).

3.2.4.2 Inhibition of Rhodamine-123 efflux

Prolonged inhibition of the efflux of R-123, a P-gp substrate, from drug resistant cells was observed following treatment with PSC 833. This compound is the most potent MDR modulator compared to verapamil and Cy A (Petriz *et al.*, 1997). Verapamil also has an inhibitory effect on daunorubicin (DNR) exclusion via non P-gp mechanisms (Versantvoort *et al.*, 1993). In cell line experiments the PSC 833/R-123 combination was the most sensitive functional P-gp assay (Broxterman *et al.*, 1996).

3.2.5 Conclusion: Investigation of MDR

The PSC 833/R-123 combination is the most sensitive and specific functional P-gp assay. This functional assay in combination with MRK16 Mab for detection of P-gp expression is the most valid method to test for P-gp dependent MDR in clinical samples. This is the method used in the majority of the experiments performed during this research project.

CHAPTER 4

MATERIALS AND METHODS

4.1 Materials

Peripheral blood mononuclear cells (PBMC) were obtained from the renal transplant patients at Walsgrave Hospital, Coventry, UK and served as the main material for all the investigations. Blood was taken from each patient in the morning prior to ingestion of the next dose of Cy A or FK506. In all cases patients had provided prior written consent. Information and consent forms were approved by the local ethics committee (See Appendix 1).

A total of 71 patients were entered into the study. Access to the patients' medical records, either through individual case notes or from data held on the Proton computer system at the renal unit at Walsgrave Hospital, Coventry was obtained. To ensure anonymity, patients are referred to only by serial number, with the code held by the author.

The majority of these patients were on "triple therapy": Cy A *or* FK506 with azathioprine and prednisolone. Ages ranged from 23 to 73 years (mean 40 years) with male and female. Samples were taken 1-77 months post transplant (mean 19 months). The characteristics of the patients are summarised in Table 4.1

Table 4.1. Patient characteristics.

Pt No.	Age (years)	Sample date month/yr	Early/ Late	Triple therapy	Steroid therapy	Trough blood Creatinine. Level µmol/l
22	35	12/96	E	Y	Y	258
18	26	8/96	E	Y	Y	153
38	57	1/97		Y	Y	146
21	39	10/96	L	Y	Y	220
19	45		L	Y	Y	130
14	34		L	Y	Y	200
29	39	12/96		Y	Y	118
26	24			N	Y	102
						97
20	23	1/97	L			158
25	32			Y	Y	200
2	49	12/96	L	Y	Y	140
27	37	1/97	L	Y	Y	227
17	51	10/96	E	Y	Y	125
8	27	10/96	E	N	Y	190
23	43	10/96	E	N	Y	219
34	29	2/97		Y	Y	170
12	48	10/96	L	N	N	130
6	32	10/96	L	Y	Y	181
9	40	1/97	E	N	Y	118
28	27	11/96				152
1	48	8/96	L			191
31	51	12/96	L	N	N	139
11	44	11/96	L	N	Y	156
24	56	1/97	L	Y	Y	139
7	25	12/96	E	Y	Y	113
37	51	11/96	E	Y	Y	139
7	25	11/96	E	Y	Y	439
36	54	12/96	L	Y	Y	155
30	31	1/96	E	N	Y	80
3	47	10/96	E	Y	Y	163
32	33	1/97	E	Y	Y	170

4	61	10/96	E	Y	Y	110
35	73	10/96	L	N	N	1000
39	31	12/96	E	Y	Y	224
40	40	08/96	L	N	N	156
41	62	02/98	L	Y	Y	116
42	57	02/98	L	N	N	123
43	43	02/98	L	N	N	201
44	35	03/98	L	N	N	248
45	40	08/97	E	Y	Y	122
46	38	06/98	E	Y	Y	107
47		11/96	L	N	N	264
48		11/96	PRE	N	N	867
49	33	12/96				101
50	46	7/98	E	Y	Y	202
51	23	12/96				77
52	29	12/96				100
53	27	9/98	E	Y	Y	190
54	32	9/98	E	Y	Y	121
55	45	9/98	E	Y	Y	100
56	58	4/98	L	Y	Y	136
57	34	6/99				88
58	49	8/98	L	N	N	90
59	44	3/99	L	N	N	218
60	31	10/98	E	Y	Y	170
61	31	7/98	E	Y	Y	153
62	27	6/98	E	Y	Y	161
63	34	6/99				78
64	38	7/97	L	N	N	148

Table 4.1. Patient characteristics. Each patient number refers to an individual patient. The majority of patients were receiving triple therapy with either Cy A or FK506 plus both prednisolone and azathioprine. The serum creatinine level at the time the patient was sampled is included. Those patients referred to as 'early' had blood samples obtained within three months of their renal transplant. Those patients referred to as 'late' include all patients who had blood samples taken greater than three months after their transplant. This three month cut off was made on the basis that this is the period of 'induction' anti-rejection treatment and subsequent treatment is 'maintenance' immunosuppression.

Surrogate donor PBMC were obtained from healthy volunteers with known HLA tissue type and donor/ recipient pairing information was obtained from records kept by the Transplant Centre at Walsgrave Hospital. The HLA Class II antigens are listed for these patients and volunteers in Table 4.2.

Patient No.	Recipient HLA Class II antigen at DR locus	Donor HLA Class II antigen at DR locus
65	13(6), 12	14 (6), 7
66	11(5), 7	17(3), 11(5)
68	4, 11 (5)	4,7
69	17 (3), 13(6)	17(3), 13 (6)
70	17 (3), 14(6)	17(3), 13(6)
46	17(3), 13(6)	17 (3), 14(6)
55	4,13(6)	4,13(6)
54	11(5), 13(6)	11(5), 14(6)
50	15 (2), 17(3)	15 (2), 17(3)
60	1, 14(6)	1,13 (6)
61	17 (3), 8	17 (3), 4

Table 4.2 Recipient and donor HLA Class II antigens: Surrogate cells were used that were matched for the original donors' MHC Class II (DR) antigens. There are 11 combinations of samples that come from the transplant recipient and donor. Tissue typing gives 2 alleles at DR for each patient. The 'broad' antigens that were matched are in parenthesis, while the 'private' antigens may not be fully matched. For example, patient number 54 is matched with donor DR 5 and 6 but the less important subtype at 6 do not match identically.

Since the predominant stimulus in the MLR is obtained with mismatch of antigens at the Class II MHC locus it was necessary to match the surrogate cells for the donor cells at this locus to obtain a comparable 'physiological' allo response. The

original donor spleen cells, which would have served as stimulators in these experiments, were not available.

4.2 Methods

All operations were carried out using standard sterile conditions in a laminar flow cabinet (Gelaire BSB 4A, Flow Laboratories). The cabinets were routinely sterilised overnight by exposure to ultra-violet radiation and then washed in 70% alcohol prior to use.

4.2.1 Drugs and reagents: Cy A (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in a 9:1 ethanol/Tween (Sigma Chemicals, Poole, UK) that was subsequently diluted with an equal volume of distilled water to give a 10 mg/ml stock solution that was stored frozen in aliquots. An FK506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) solution of 0.1mg/ml was prepared and stored in a similar manner. PSC 833 (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in ethanol and subsequently diluted with an equal volume of sterile distilled water to give a 10mg/ml stock solution that was stored at 4°C. SDZ RAD (Novartis Pharmaceuticals, Basel, Switzerland) was dissolved in ethanol to give a 1mM and stored at -20°C in the dark. Prior to use SDZ RAD was diluted in RPMI to reach final concentrations in cell cultures of 0.01, 0.1, 1, 3, 10, 30, 80µg/l. Tissue culture medium was Roswell Park Memorial Institute (RPMI) 1640 (Gibco-BRL, Gaithersburg, MD USA) supplemented with 10% foetal calf serum (Gibco-BRL). RPMI was further supplemented with β-mercaptoethanol (Sigma St. Louis, MO USA) and HEPES buffer for the MLR. Lymphocyte separation medium was Lymphoprep (Nycomed Pharma, Oslo, Norway). Round-bottomed 96 well tissue culture plates were purchased from

Costar Inc (Badhoevedorp, The Netherlands). Con A was from Sigma Chemicals. ^{3}H thymidine (thy) was purchased from Amersham Radiochemicals (Amersham, UK). Rhodamine-123 (R-123) (Sigma, St. Louis, MO USA), a fluorescent dye known to be transported by P-gp, was dissolved in Phosphate Buffered Saline (PBS) to give a stock solution of 1mM which was stored in the dark at 4°C. 12 well tissue culture plates were purchased from Costar Inc (Badhoevedorp, The Netherlands). Mitomycin C (Sigma, St. Louis, MO USA) was dissolved in PBS at 1mg/ml and stored at 4°C.

4.2.2 Antibodies: Monoclonal antibodies to external epitopes of P-gp were used. These were mouse anti-human 4E3-fluorescein isothiocyanate (-FITC) conjugated, IgG2a isotype, (Signet Labs., Inc UK Glasgow, UK) and mouse anti-human MRK16, IgG2a isotype, (Kamiya Biomedical Company, Seattle, WA USA). Mouse IgG 2a isotype control antibody was purchased from Sigma, St. Louis, MO USA. Goat anti-mouse IgG-FITC was used as secondary antibody (I.D. Labs., Inc Glasgow, UK). CD4-phycoerythrin (PE) and CD8-PE were used to characterise cell subpopulations (Becton Dickinson, San Jose, CA USA). CD25-FITC was used to investigate proliferation assays by flow cytometry (Becton Dickinson, San Jose, CA USA).

4.2.3 Separation and storage of PBMC

Fresh venous blood collected into EDTA-tubes was mixed with an equal volume of culture medium and layered onto Lymphoprep and centrifuged for 30 min at 1500 rpm. The interface layer was separated, washed, and cryopreserved in culture medium with 10% dimethylsulphoxide.

4.2.4 PBMC culture and incorporation of ^{3}H thymidine: PBMC aliquots from an initial 32 patients were thawed rapidly, washed twice with culture medium to free them of dimethylsulphoxide and a viable count performed. If viability was below 90% (which was very rare) the cells were discarded. The cell concentration was adjusted to 10^{6}/ml and the suspension placed in 200µl aliquots into the wells of tissue culture plates. 6 wells were reserved as negative controls: to a further 6 positive controls was added Con A alone to a final concentration of 5 µg/ml: to the remaining wells, Con A plus graded concentrations of the two drugs were added, in triplicate. Final concentrations of Cy A were 1, 3, 10, 30, 100 and 300 µg/l and of FK506 were 0.1, 1, 3, 10, and 30 µg/l. Similarly, cells from further patients were treated in the same way but graded doses of SDZ RAD either alone or in combination with FK506 were established. The final concentration of SDZ RAD were 0.01, 0.1, 1, 3, 10, 30, 80µg/l.

PBMC from a further 14 patients were treated in the same way except that 2µM PSC 833 was added along with either Cy A (n=9) or FK506 (n 5). Controls with PSC 833 only were also established. After 48h culture, 1µCi/ml ^{3}H thymidine was

added to each well, the culture continued overnight to allow incorporation, and then the cells harvested to glass fibre pads (Meltilex™, WALLAC®, Turku, Finland), washed, and the incorporated ^{3}H counted using a scintillation counter (WALLAC®). The concentration of drug required to inhibit 50% of ^{3}H-thy incorporation (ID_{50} value) were established for all patients for both drugs by interpolation from graphs of incorporation against drug concentration.

4.2.5 Mixed Lymphocyte Reaction (MLR): PBMC from the surrogate donor/recipient combinations were thawed in the same way as described for the proliferation experiments. PBMC from the donor were treated with 20µl of mitomycin C (1mg/ml) and washed thoroughly prior to co-culturing with recipient PBMC. Graded doses of 20µl of each of FK506 and/or SDZ RAD were added to the cell cultures. Controls were established with no drugs. The cell cultures were incubated for 96 hours at 37°C in 5% CO_2 in air. ^{3}H-thymidine was then added and left overnight to incorporate into the cells prior to counting. To check PBMC viability Con A was added to replicate control cultures at 48 hours.

4.2.6 P-gp Expression: The percentage of total cells expressing surface P-gp was determined by immunofluorescence techniques in those patients that were defined as either drug sensitive or drug resistant following the PBMC cultures. Briefly, cells were washed twice in ice-cold PBS. Aliquots of 50µl (5×10^5 cells) were incubated with either 40µl of the undiluted 4E3-FITC Mab (5µg/assay) or 50µl of 1:10 dilution of MRK16 Mab (2.5µg/assay), as per manufacturers instructions, for 30 minutes on ice. After incubation the cells were washed thoroughly in PBS and the secondary antibody (50µl of 1:25 dilution of Goat anti-Mouse) added in the

case of MRK16, and incubated for 15 minutes on ice. Further washes in ice-cold PBS were performed and the samples were resuspended in PBS and kept at 4°C in the dark until analysis. Control isotype antibodies at the same concentration as the test antibodies were treated in the same way.

4.2.7 Rhodamine Efflux: Aliquots containing approximately $5x10^5$ cells loaded with R-123 by incubating for 30 minutes at 37°C with R-123 (final concentration 2μM). After loading the cells were washed and resuspended in complete RPMI-1640 in the presence or absence of PSC 833 (final concentration 2μM) and incubated for a further 4 hours. At time points 0 and 4 hours a sample was taken for study and the cells washed and kept in ice-cold PBS in the dark until analysis. In some cases the samples were then incubated with anti-CD4-Phycoerythrin (PE) and CD8-PE Mab to examine which subsets of T cells are actively pumping the dye. Samples were analysed on a FACStar flow cytometer (Becton Dickinson), equipped with a 488nm argon-ion laser and standard optical set-up. 10000 events were analysed with Consort 30 software (Becton Dickinson) and subsequently analysed with DAKO Flowmate II for Windows software. Fluorescence was measured on a four-decade logarithmic scale. Manual gating via scattergram on Forward Scatter (cell size) versus Side Scatter (cell granularity) identified the cell population of interest. P-gp staining results are expressed as mean fluorescence intensity (MFI), and the percentage of cells actively effluxing R-123 is estimated from histograms obtained for each sample

4.2.8 Cell Line: The adriamycin drug-resistant breast carcinoma cell line MCF-7/ADR (originally from European Collection of Animal Cell Cultures, CAMR, Porton Down, Salisbury UK), were maintained in house for use as the positive controls in the rhodamine efflux and P-gp staining experiments.

4.2.9 T cell activation marker

CD25-FITC was used in flow cytometry studies of T cell activation. Con A stimulated cell cultures of PBMC were established in 4 patients as before. Graded doses of SDZ RAD and varying fixed doses of FK506 were added at the beginning of the cultures. The cells were incubated for 48 hours prior to harvesting and labelling with CD25-FITC.

4.2.10 Dual staining of R-123 loaded cells with lineage markers

Following R-123 loading and subsequent incubation to allow efflux to occur the cells were harvested and then counter-stained with either CD4-PE or CD8-PE monoclonal antibodies and kept on ice in the dark until analysis.

4.2.11 Interpretation of histogram data

Histograms were obtained for R-123 efflux and P-gp expression by flow cytometry. The data can be arranged as a histogram of cell number against fluorescent intensity. This is best illustrated by referring to Figure 4.1

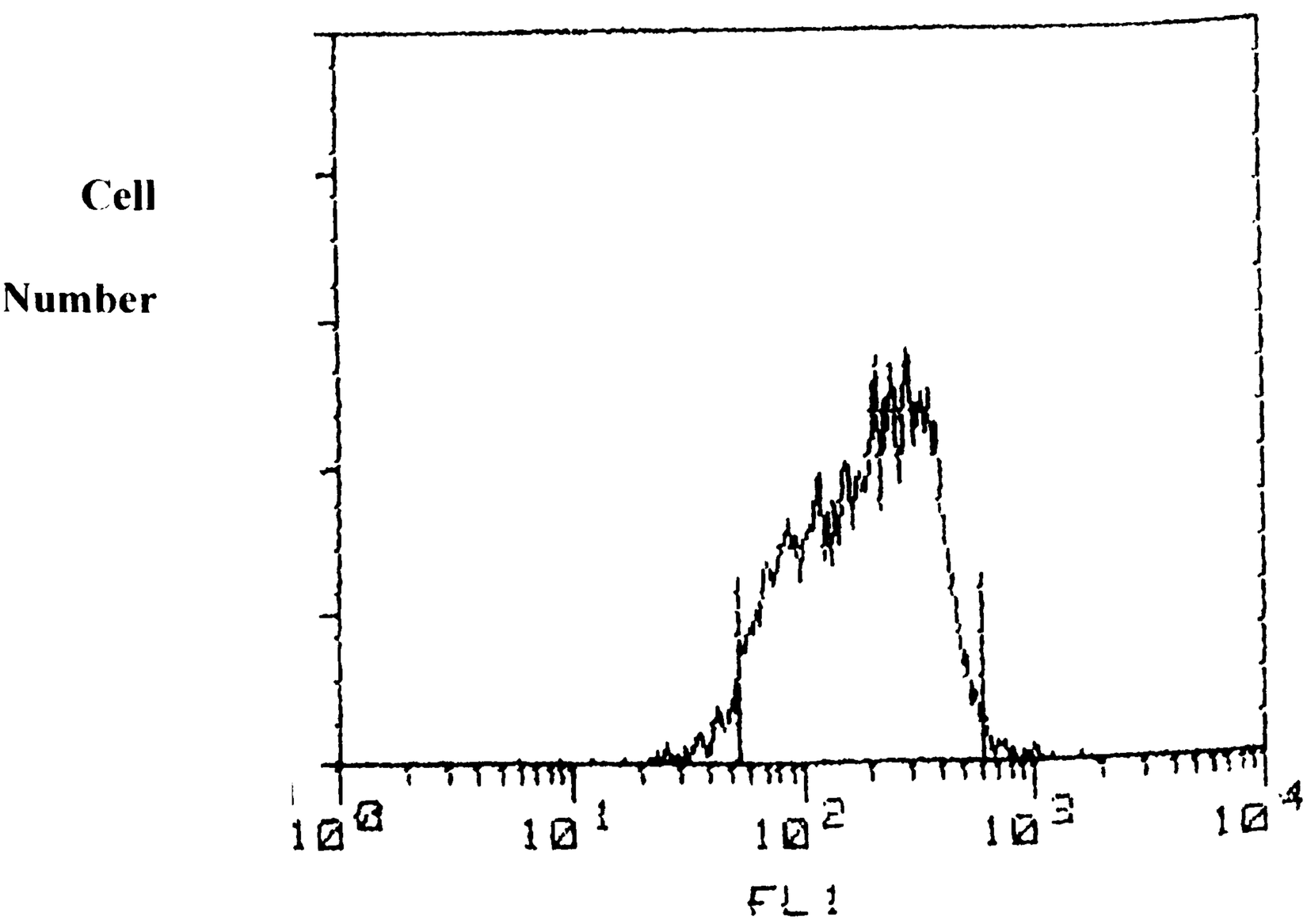


Figure 4.1 Histogram of Rhodamine loading of PBMC. Cells from a single patient were incubated for 30 minutes with R-123 at 2μM. The cells were then washed in ice-cold PBS and kept on ice until FACS analysis

Analysis of efflux curves is illustrated with reference to Figure 4.2.

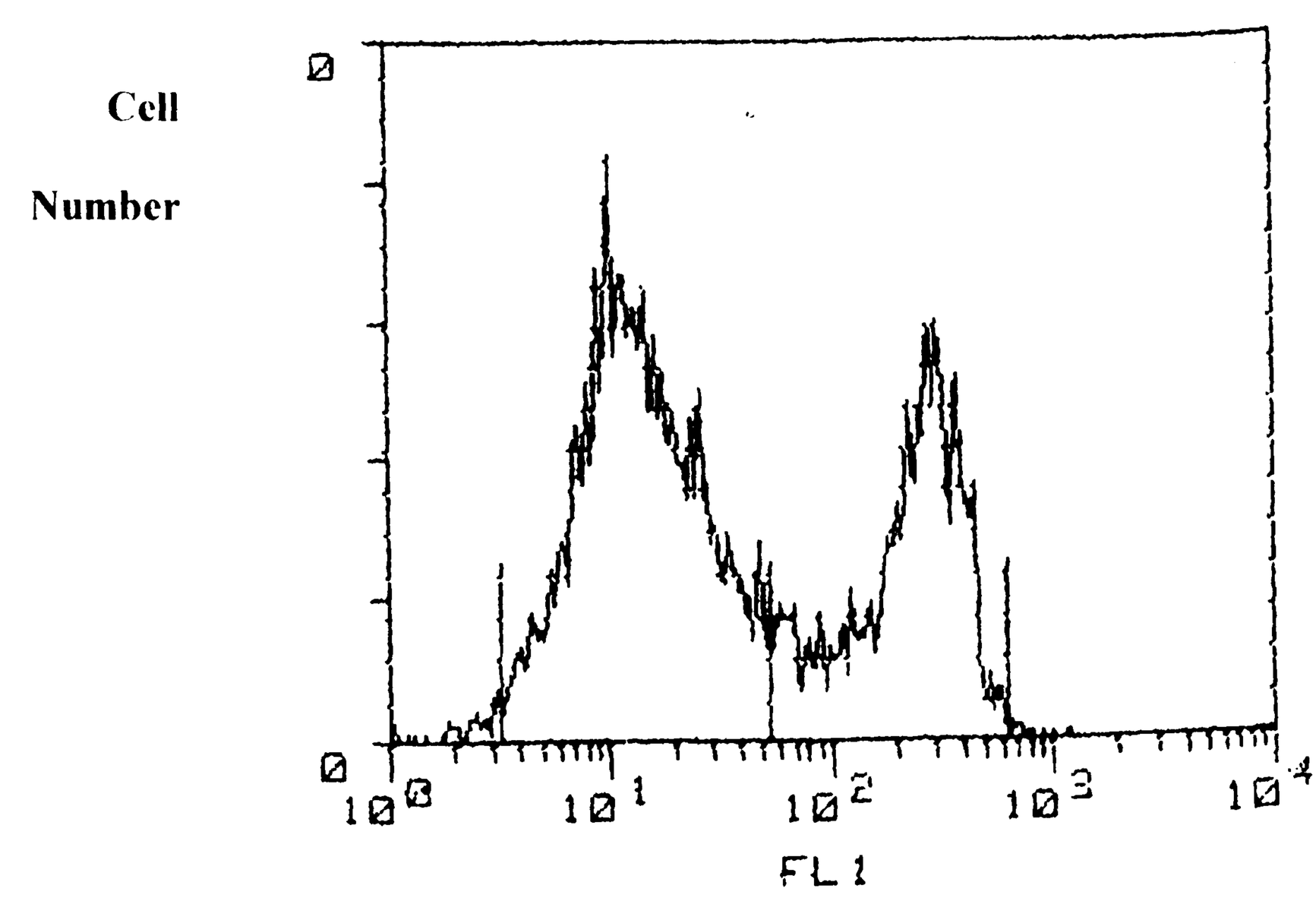


Figure 4.2 Histogram of Rhodamine efflux. PBMC from a single patient were incubated with 2μM R-123 for 30 minutes with a subsequent 6-hour incubation period in the absence of the dye. Three markers have been manually inserted to separate the cells into two populations based on the level of fluorescence.

The number of cells per population can be estimated from the percentage of cells gated in each group. During this experiment 62.0% of cells were in the effluxing group and 33.3% in the retaining group. The percentage of gated cells in each population can then be calculated: Total number of cells gated and therefore

studied is 95.3%. Percentage of cells within effluxing pool is 62.0/95.3 0.65 X 100 i.e. 65% are in the effluxing pool and 35% within retaining pool.

These calculations were repeated for each patient sample tested. Therefore it was possible to estimate the percentage of cells in the effluxing population for all patients in both the high and low sensitivity groups.

4.2.12 Drug Blood levels

Pre-dose (trough) Cy A and FK506 levels were measured in whole blood using a system from Abbott (Maidenhead, UK).

4.2.13 Statistical Analysis

Tests of significance were carried out using the "Astute" software package (University of Leeds, UK) and "Excel" (Microsoft). Spearman Rank was used to test the correlation of lymphocyte sensitivity to Cy A and FK506. The Mann-Whitney-U test was used to examine significance of selective lymphocyte resistance to Cy A and FK506 depending on the drug the patient was receiving for treatment. In all other cases the mean values of groups were tested for significance by paired t-tests.

CHAPTER 5

RESULTS

5.1. Assessment of lymphocyte sensitivities to calcineurin inhibitors

Introduction

The range of sensitivities of peripheral blood mononuclear cells (PBMC) from renal transplant recipients to calcineurin inhibitors, Cy A and FK506, in *in vitro* cultures were established. Patients were subsequently stratified as highly sensitive, moderately sensitive or of low sensitivity to both Cy A and FK506. These experiments investigated any differences in sensitivities to either drug in individual patients.

Results

Incorporation of ^{3}H thymidine into Con A stimulated patient PBMC was progressively inhibited by increasing concentrations of both drugs. The inhibitory effects of the two drugs varied from patient to patient, both in the nature of the response and the level of sensitivity to the drugs. Examples of the response of Con A stimulated PBMC from 4 renal transplant patients are shown in Figure 5.1. Two (2 and 21), representative of the majority of the patients, showed essentially complete inhibition with both drugs. The other two (3 & 4), representative of 5 patients, showed a rather different response. With these, even at the highest concentrations of drug used, inhibition was incomplete, only just exceeding 50%.

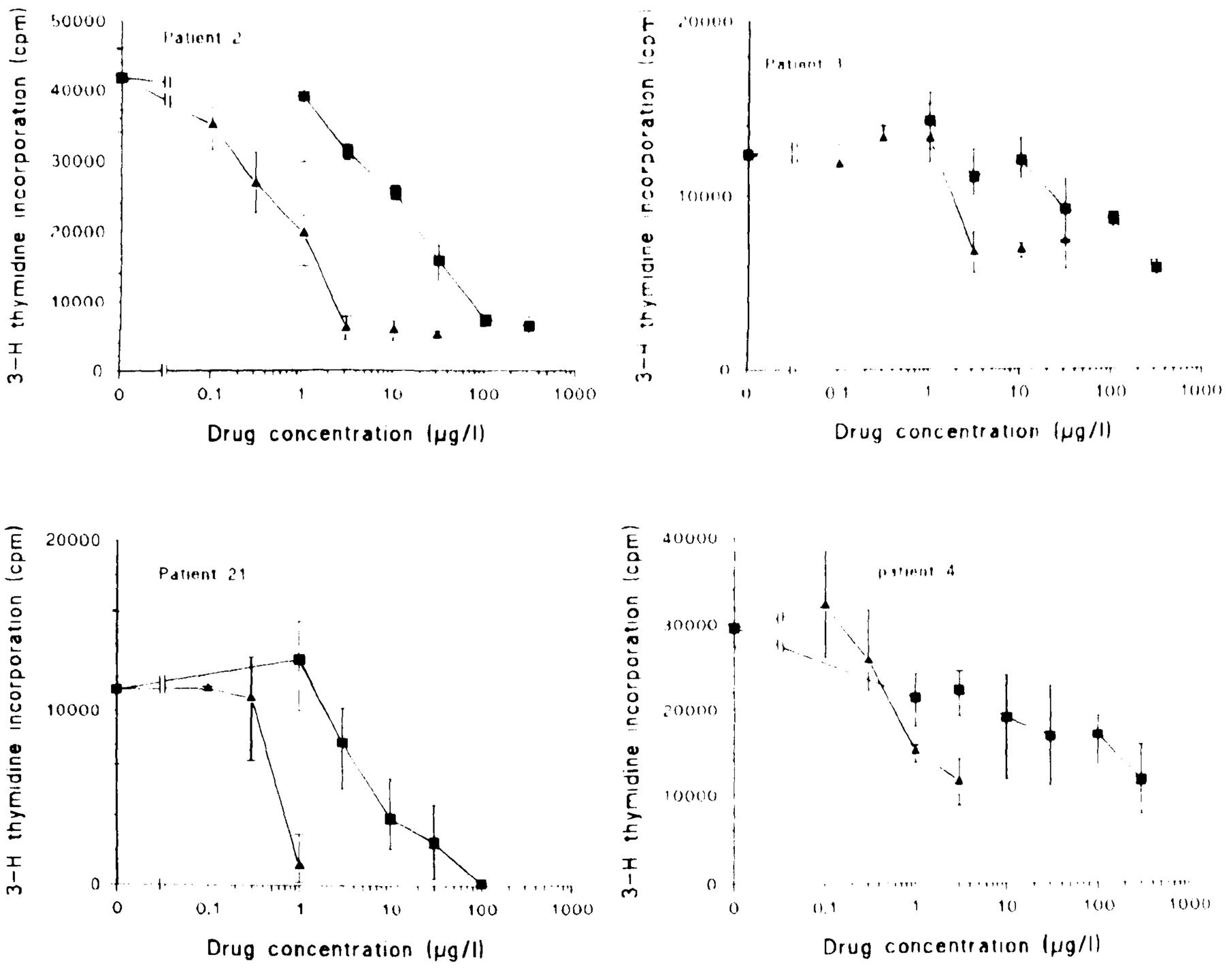


Figure 5.1 Representative dose response curves in 4 patients of the effects of Cy A and FK506 on Con A stimulated PBMC. PBMC from each single patient were incubated in the presence or absence of Cy A (■) and FK506 (▲), as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars represent standard deviation around the mean.

The ID_{50}s are presented for 32 patients in Table 5.1. Where there were sufficient PBMC for duplicate assays of the same sample, performed on separate occasions, the ID_{50}s generated agreed within a 2-fold range, and the mean values where duplicate assays were done are quoted in the table. In the cases of some patients repeat samples were taken and the ID_{50} values obtained for these repeat samples are quoted separately.

Table 5.1. ID_{50s} for Cy A and FK506 following Con A stimulation of PBMC

Patient Number	ID_{50} for Cy A µg/l	ID_{50} for FK506 µg/l	Ratio Cy A: FK ID_{50s}
1	28.5 (2)[b]	nd	
2	14	0.65	21.5
2 (rep)[d]	45 (2)	0.65 (2)	69
3	85 (2)	0.8	106
4	100 (2)	5.5	18
4 (rep)	30	5	6
6	20	0.5	40
7	32	0.6	53
8	16	2.3	7
9	23 (2)	0.7 (2)	33
9 (rep)	28	0.37	76
11	30 (2)	0.4 (2)	75
12	21 (2)	0.39 (2)	54
13	40 (2)	0.68 (2)	59
14	9	0.27	33
17	30	0.8	38
17 (rep)	15	0.6	25
18	60	4	15
18 (rep)	2.2	0.17 (2)	13
19	5	0.15	33
20	12	0.3	40
21	4.6	0.6	7.7
22	1.5	0.085	18
23	17	1.4	12
24	30	1.5	20
25	14	0.45	31
26	28	2	14
26 (rep)	12	0.35	34
26 (rep)	13	0.55	24
26 (rep)	11.5 (2)	0.44	26

27	15	0.2	75
27 (rep)	22.5 (2)	nd	
28	27.5 (2)	0.24 (2)	115
29	9.5	0.16	59
30	100	10	10
30 (rep)	50	0.55 (2)	91
31	30	0.5	60
34	20 (2)	0.4 (2)	50
35	100	0.5	200
36	40	1.2	33
37	34 (2)	0.5 (2)	68
38	2.5	nd	
mean	**29**	**1.2**	**30**
SD	**25**	**1.9**	
median	**25**	**0.55**	

Table 5.1.ID_{50s} for Cy A and FK506 following Con A stimulation of PBMC ID50s were obtained in proliferation assays by interpolating the dose-response curves obtained following the addition of Cy A and FK506. Where there were sufficient PBMC for duplicate assays of the same sample, performed on separate occasions, the ID_{50}s generated agreed within a 2-fold range, and the mean values where duplicate assays were done are quoted in the table. In the cases of some patients repeat samples were taken and the ID_{50} values obtained for these repeat samples are quoted separately.

Blood levels of Cy A and FK506 were taken prior to the next dose from all patients receiving the drugs as part of their anti-rejection regime. The drug level obtained and the ratio of trough blood level: ID_{50} are shown in Table 5.2

Table 5.2 Trough blood levels of Cy A and FK506 and ratio of blood level :ID_{50}

Patient No.	Cy A blood levels[a] ng/ml	ratio blood level:ID_{50} (Cy A)[a]	FK506 blood levels[a] ng/ml	ratio blood level:ID_{50} (FK506)
1	Nd[c]		nd	
2	128	9		
2 (rep)[d]	166	3.7		
3	234	2.8		
4	170	25		
4 (rep)	nd			
6	131	6.5		
7	200	6.3		
8			13.5	5.9
9			17.4	25
9 (rep)	nd			
11	155	5.2		
12	118	5.6		
13	nd			
14	nd			
17	nd			
17 (rep)			11.8	19.7
18			8.6	2.2
18 (rep)	227	103		
19	117	23		
20	176	15		
21	210	46		
22			24.6	290
23			11.1	8
24			15.8	10.5

25			11.3	25
26	nd			
26 (rep)			9.5	27
26 (rep)			11.2	20
26 (rep)	296	26		
27	179	12		
27 (rep)	1000[e]	44.4		
28	169	61		
29	nd			
30	457	9		
31	137	4.5		
34		14.1	35	
35	171	1.7		
36	131	3.3		
37	nd			
38	159	64		
mean	**230**	**14**	**49**	.
SD	**190**	**5**		.
Median	**170**	**12**		.

Table 5.2 Trough blood levels of Cy A and FK506 and ratio of blood level :ID_{50}

Footnotes: [a]Patients were treated with Cy A or FK506: hence a blank in one column indicates that the patient was treated with the other drug.

[b]Indicates mean of 2 determinations: otherwise single determinations.

[c]nd = not done

[d]Indicates determination with a repeat sample (i.e. taken on a separate occasion)

[e]minimum value

The values of the ID_{50}s for both drugs varied widely from patient to patient, with a 50-fold range. However, when the correlation between the two sets of ID_{50} values was analysed by the Spearman Rank method it was found to be significant ($p = 0.0001$). Figure 5.2A shows the regression of the ID_{50} for FK506 against that

for Cy A. The ratio of the mean values for the ID_{50} for Cy A and FK506 was 24. This correlation conceals some considerable variation between patients in the ratio of the two ID_{50}s, from less than 10 (e.g. patients 4, 8 and 21) to 100 or more (patients 3, 28 and 35). This indicates that individual patients are relatively resistant to one or the other drug.

To test whether this resistance was selective, i.e. patients' PBMC were relatively resistant to the drug used for treatment, the ratio of the ID_{50}s was compared for the patients treated with either Cy A or FK506. Figure 5.2B shows that patients' PBMC were relatively resistant to the drug used for treatment. The median value for the ratio of ID_{50} for Cy A to ID_{50} for FK506 for patients treated with Cy A was 47 and for patients treated with FK506 was 24: p value for the comparison by Mann-Whitney U test was 0.02.

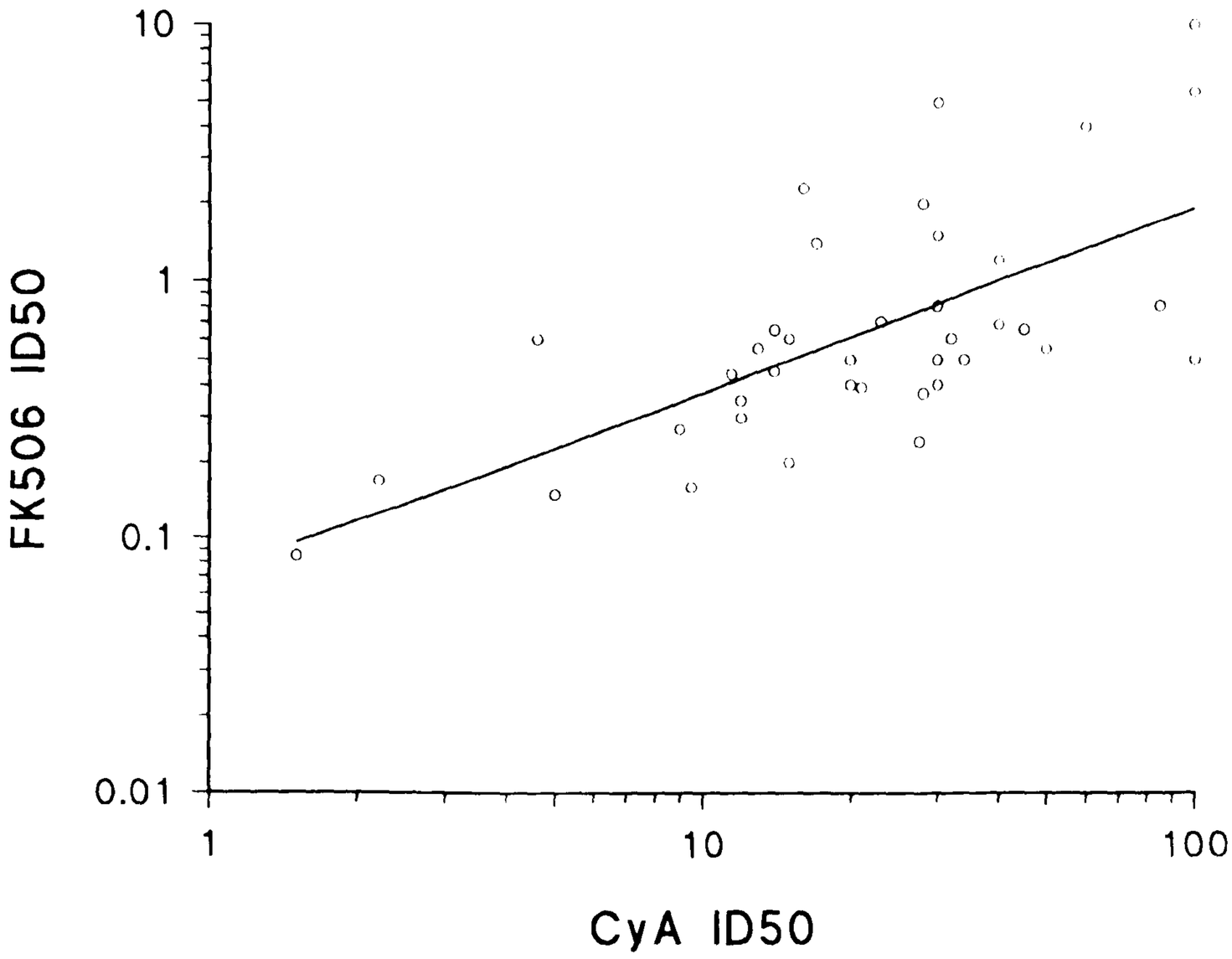


Figure 5.2A Correlation of values of ID_{50}s for Cy A and FK506 for all patients. ID_{50}s (μg/l) for Cy A and FK506 were obtained for 32 patients. There is a significant correlation of ID_{50}s obtained for both drugs as tested by Spearman Rank p=0.0001.

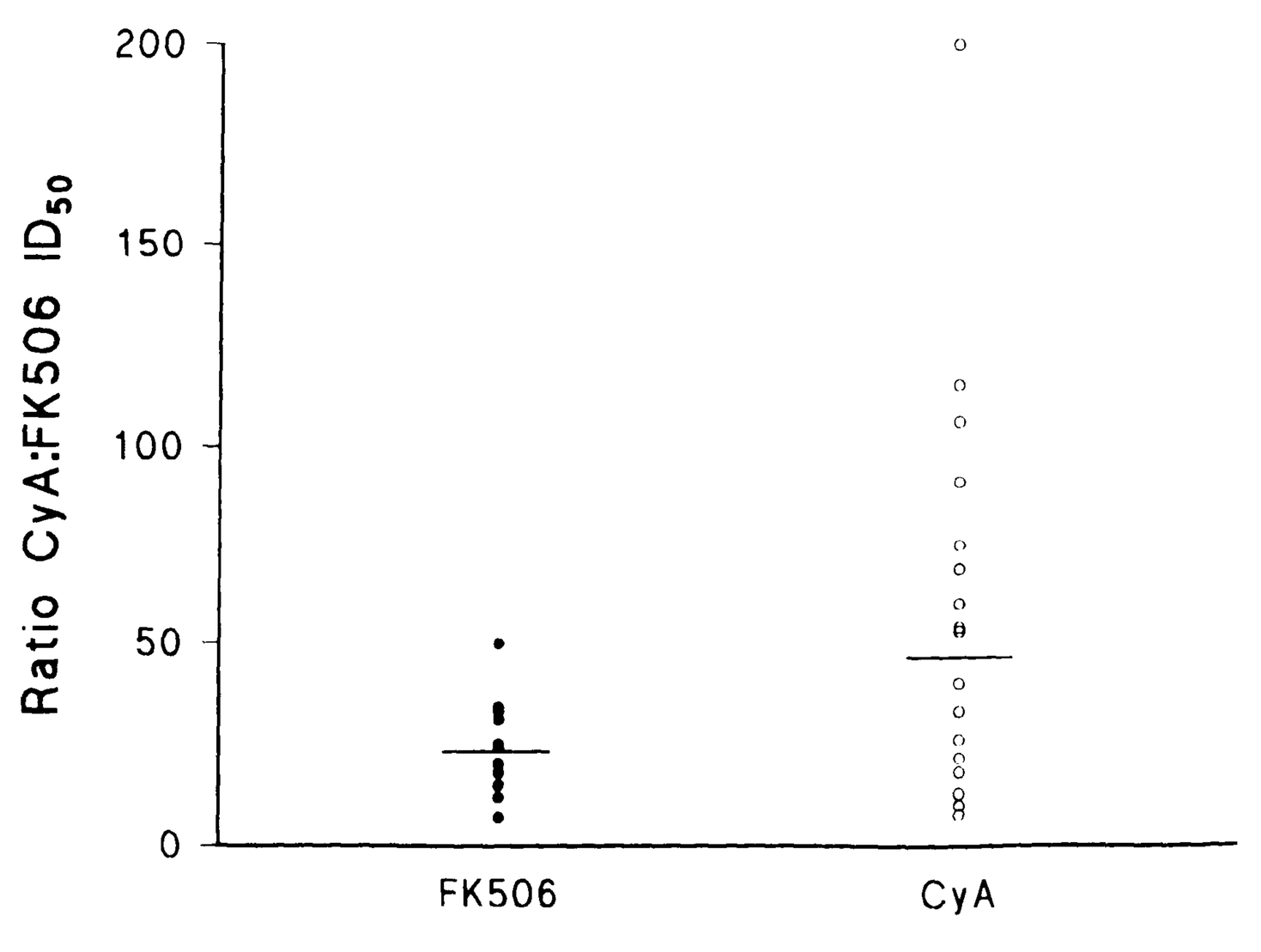


Figure 5.2B Ratio of ID_{50}s for the two drugs in patients treated with either one or other drug. The ratios of the ID_{50}s obtained Cy A and FK506for each drug were analysed against the drug used for treatment. Patients treated with FK506 were relatively more resistant to FK506 in these experiments. This was also the case with patients being treated with Cy A.

Since both Cy A and FK506 have significant toxic side effects, an aim of therapy must be to use the minimum dose of drug consistent with preventing rejection. As can be seen from Table 5.2, the ratio of mean trough blood level to ID_{50} was about 30 for Cy A and about 49 for FK506. Again comparing means conceals wide individual variations. For example, for patients treated with Cy A the ratio may be less than 3 (patients 3, 30 & 35) or more than 60 (18, 27 and 38): likewise for FK506, 18 and 22 respectively have low and high ratios (18 had been transferred from FK506 to Cy A). If the optimum dose of immunosuppression were being given, one might expect that the sensitivity to the drug should correlate with the blood level: but analysis by the Spearman Rank method shows no correlation whatever (data not shown). Comparing ID_{50} for either drug between patients who did or did not suffer acute rejection, no difference was found. However, the number of patients with biopsy proven or treated rejection was low (7). There was no correlation between sensitivities and trough blood creatinine levels. The mean serum creatinine level was 186.8 μmol/l (SD 154.8 range 97-1000; median 153). The majority of patients were receiving standard 'triple' anti rejection therapy including either cyclosporin or FK506, azathioprine and prednisolone. There was no difference in sensitivities in those patients receiving steroids or not receiving steroids. Time since transplant was arbitrarily divided into early and late groups. Those patients who were within 3 months of receiving the allograft are defined as early. All other patients are referred to as late (See Table 4.1). Again there was no difference in sensitivities of lymphocytes between early and late groups.

Patients were allocated into three groups based on the ID_{50}s obtained for the immunosuppressive drugs. These groups were high, medium or low sensitivity. Table 5.3 shows the patients allocated to the high and low sensitivity groups. The remainder of the patients were of medium sensitivity and were not studied further.

High sensitivity group

Patient No.	ID_{50} for Cy A (μg/l)	ID_{50} for FK506 (μg/l)
22	1.5	0.085
18	2.2	0.17
38	2.5	
21	4.6	0.6
19	5.0	0.150
14	9.0	0.270
29	9.5	0.160
26 (a)	11.5	0.440
26 (b)	12.0	0.350

Low sensitivity group

Patient No.	ID_{50} for Cy A (μg/l)	ID_{50} for FK506 (μg/l)
2	45.0	0.650
3 (a)	65.0	1.0
3 (b)	85.0	0.80
7	100.0	10.0
32	100.0	
30	100.0	10.0
4	100.0	10.0
35	100.0	0.50

Table 5.3. Selection of patients into low and high sensitivity groups. Patients were stratified into groups depending on their sensitivities to Cy A and FK506 in proliferative assays.

5.2 Investigation of resistance mechanisms to immunosuppressive drugs in PBMC from renal transplant patients

Introduction

P-glycoprotein-170 (P-gp), the product of the multidrug resistance (MDR1) gene in man is expressed on lymphocytes from normal volunteers (Pilarski *et al*, 1995). The hypothesis that highly sensitive lymphocytes had little demonstrable P-gp expression or functional activity, whereas those cells that were relatively resistant to both drugs had high levels, leading to a reduction in the intracellular concentration and, therefore, the efficacy of the drug was explored. Both Cy A and FK506 are substrates for P-gp (Saeki *et al.*, 1993) (see Sections 1.6.6).

5.2.1 Demonstration of functional activity of the MDR1 phenotype, P-glycoprotein-170, on unstimulated lymphocytes

To investigate the functional activity of P-gp cells are loaded with a fluorescent P-gp substrate, in this case Rhodamine-123 (R-123), and then incubated in R-123-free medium for between 4-6 hours. Retention of R-123 within the cells is then analysed using flow cytometry. Those cells retaining the dye have low levels of P-gp functional activity and those that expel the dye having high levels of activity.

Results

During preliminary experiments PBMC from normal volunteers were used. Patients cells were limited resources and were studied when the experimental conditions were optimized. A histogram plot of cell numbers against Mean Fluorescent Intensity (MFI) at time 0 hour (i.e. following loading with R-123) is shown in Figure 5.3. The cells of interest were gated manually as described in Section 4.2.7. During this analysis 5000 events were collected although in most experiments 10000 cells were collected. The histogram demonstrates a normal distribution of R-123 retention within the cells. There is an approximate 10-fold difference between cells at either end of the curve.

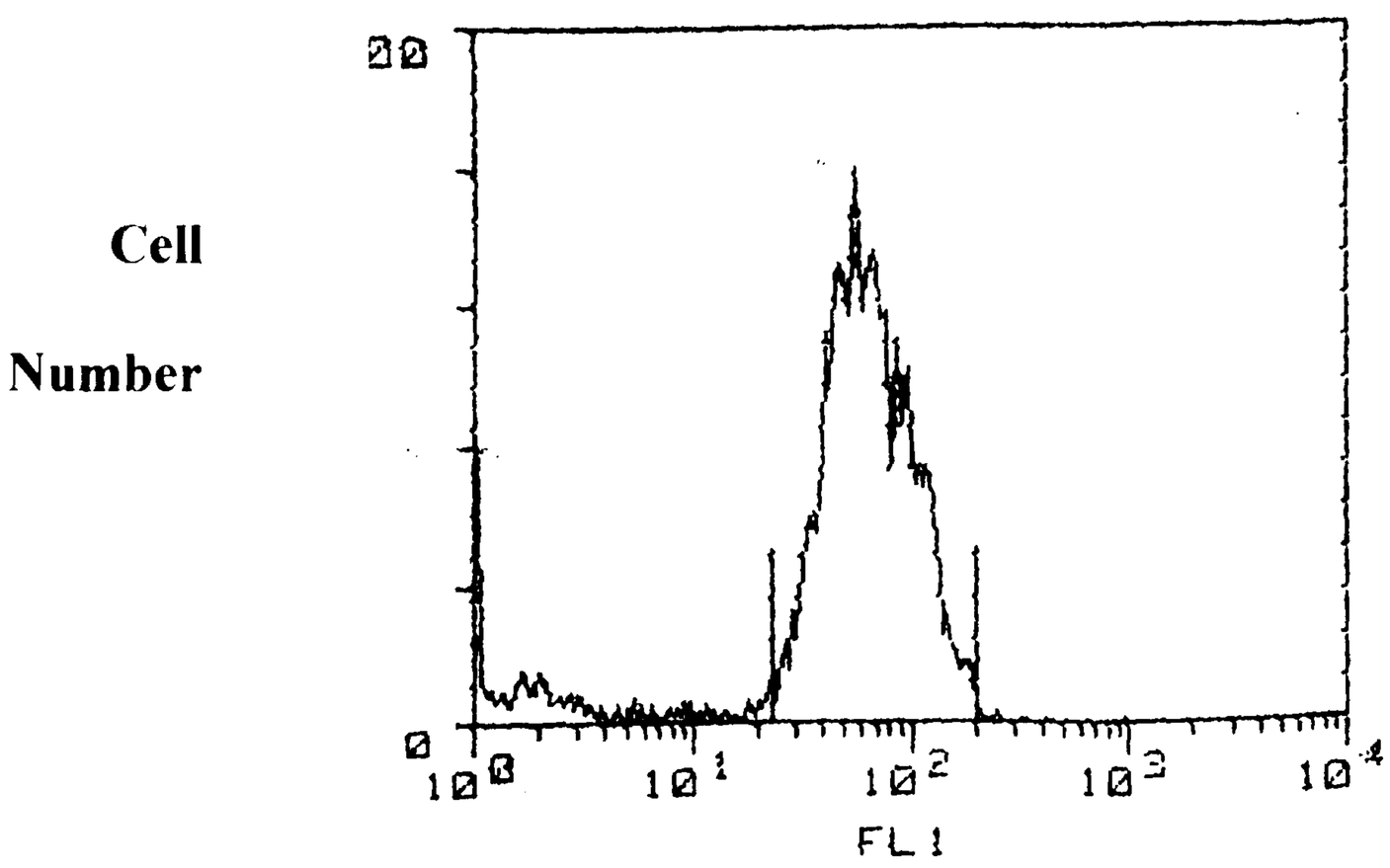


Figure 5.3 Histogram of R-123 loading of PBMC. Cells from a single patient were incubated for 30 minutes with R-123 at 2μM. The cells were then washed in ice-cold PBS and kept on ice until the FACS analysis.

Cells from a further volunteer were then loaded with R-123 and incubated in R-123 -free medium for up to 6 hours (Figure 5.4Aand B)

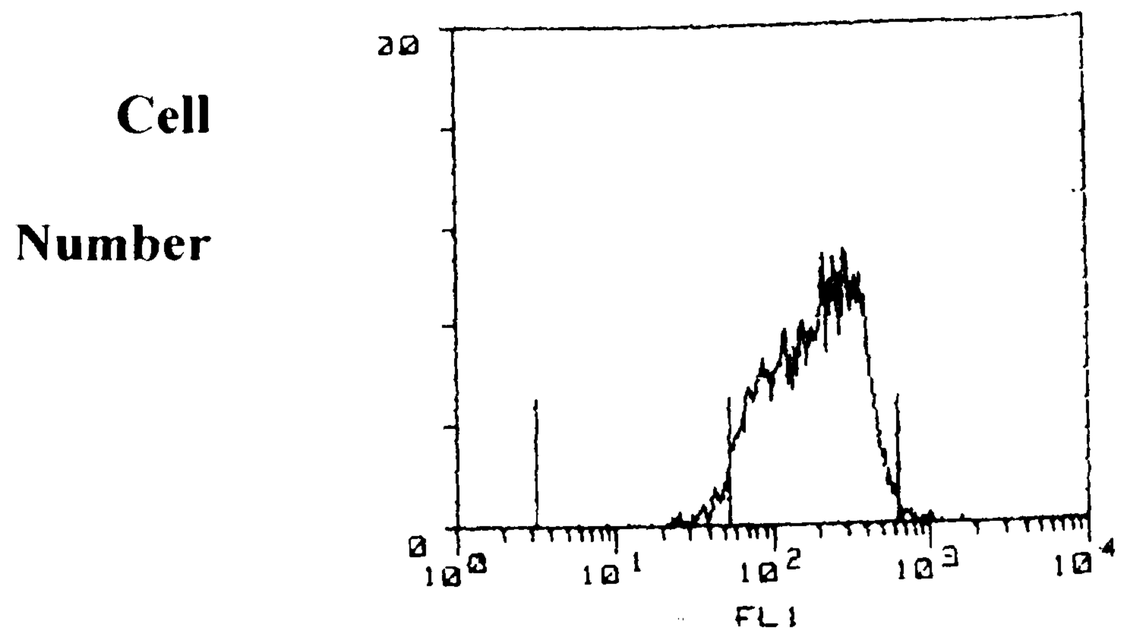


Figure 5.4A Histogram of R-123 uptake at 0 hours. Cells from a single patient were incubated for 30 minutes with R-123 at 2μM. The cells were then washed in ice-cold PBS and kept on ice until FACS analysis. Markers have been inserted manually to separate cell populations based on the level of fluorescence.

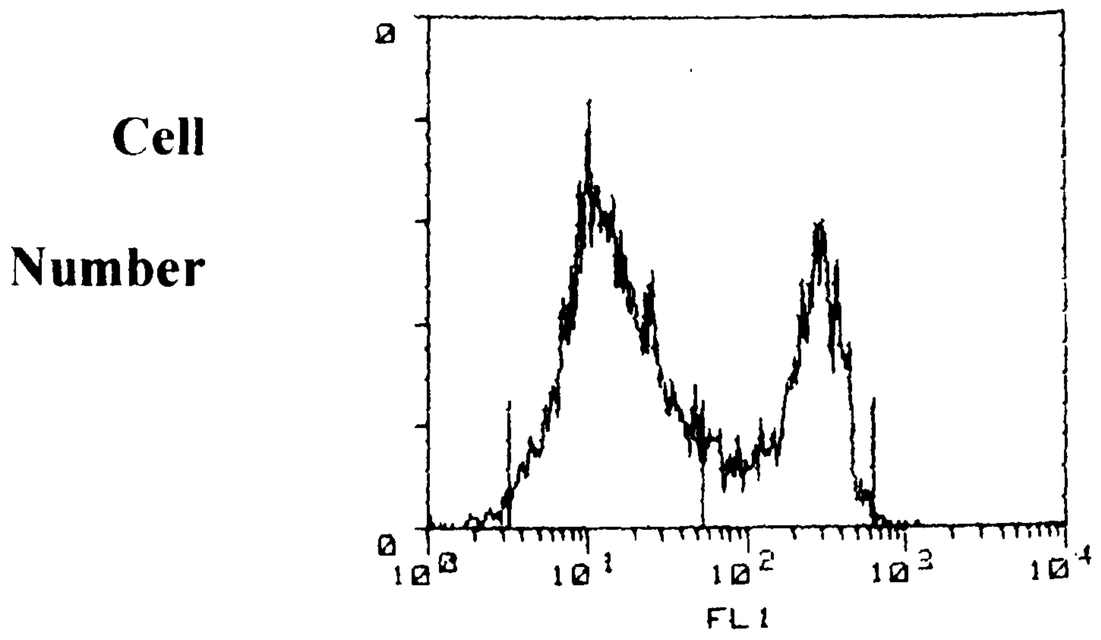


Figure 5.4B Histogram of R-123 uptake at 6 hours. Cells from a single patient were loaded with 2μM R-123 for 30 minutes and subsequently incubated in R-123-free medium for 4 hours. Markers have been placed manually to separate cells based on the level of fluorescence.

Two populations of cells have manifested during the incubation period. The histogram has two peaks. The majority of cells have effluxed the R-123 fluorochrome as evidenced from the low levels of fluorescence. The second group of cells have retained the dye and the MFI for this population is as high as the loading sample taken at time 0 hour. For each experiment the data can be further analysed. This histogram serves as a model for the analyses that were performed for each experiment as described in section 4.2.11. As can be seen from Figure 5.4B a marker has been introduced manually to form two gated populations. The histogram data includes an estimate of the Mean fluorescent Intensity (MFI) of the cells in each gated population. In this example the MFI for the cells that retained R-123 was 239.38 and the MFI for the cells that effluxed R-123 was 16.83. The percentage of cells in each of the gated populations can be calculated. The percentage of cells that have effluxed R-123 was calculated for each patient tested. The results of these experiments are shown in Table 5.4.

Patient No.	ID_{50} for Cy A (μg/l)	ID_{50} for FK506 (μg/l)	Percentage of cells effluxing R-123
High Sensitivity Group			
22	1.5	0.085	49
18	2.2	0.17	35
38	2.5		31
21	4.6	0.6	37
19	5	0.15	22
14	9	0.27	37
29	9.5	0.16	26
26	11.5	0.440	28
Mean	**5.7**	**0.28**	**33.1**
SD	**3.79**	**0.19**	**8.4**
Low Sensitivity Group			
2	45	0.65	48
3	85	0.8	30
13	40	0.68	41
36	40	1.2	32
30	100	10	27
4	100	5.5	25
35	100	0.5	64
Mean	**73**	**2.8**	**38.1**
SD	**30**	**3.7**	**14**

Table 5.4. Individual drug sensitivity and level of R-123 efflux from PBMC. The percentage of cells that effluxed R-123 was established in patients in high and low sensitivity groups. Patients were assigned these groups based on the sensitivity of PBMC to Cy And FK506. The mean percentage of cells effluxed in each group was calculated. t-Test comparing percentage of cells effluxing in high and low resistance groups; $p=0.42$.

It can be seen from Table 5.4 that in all patients drug pumping is present.

Although the mean value for percentage of cells effluxing the dye was higher in

the low sensitivity group compared to the highly sensitive group (38.1:33.1) there was no significant statistical difference between the two groups.

5.2.2 Dual labelling experiments to investigate T cell subclasses responsible for R-123 efflux

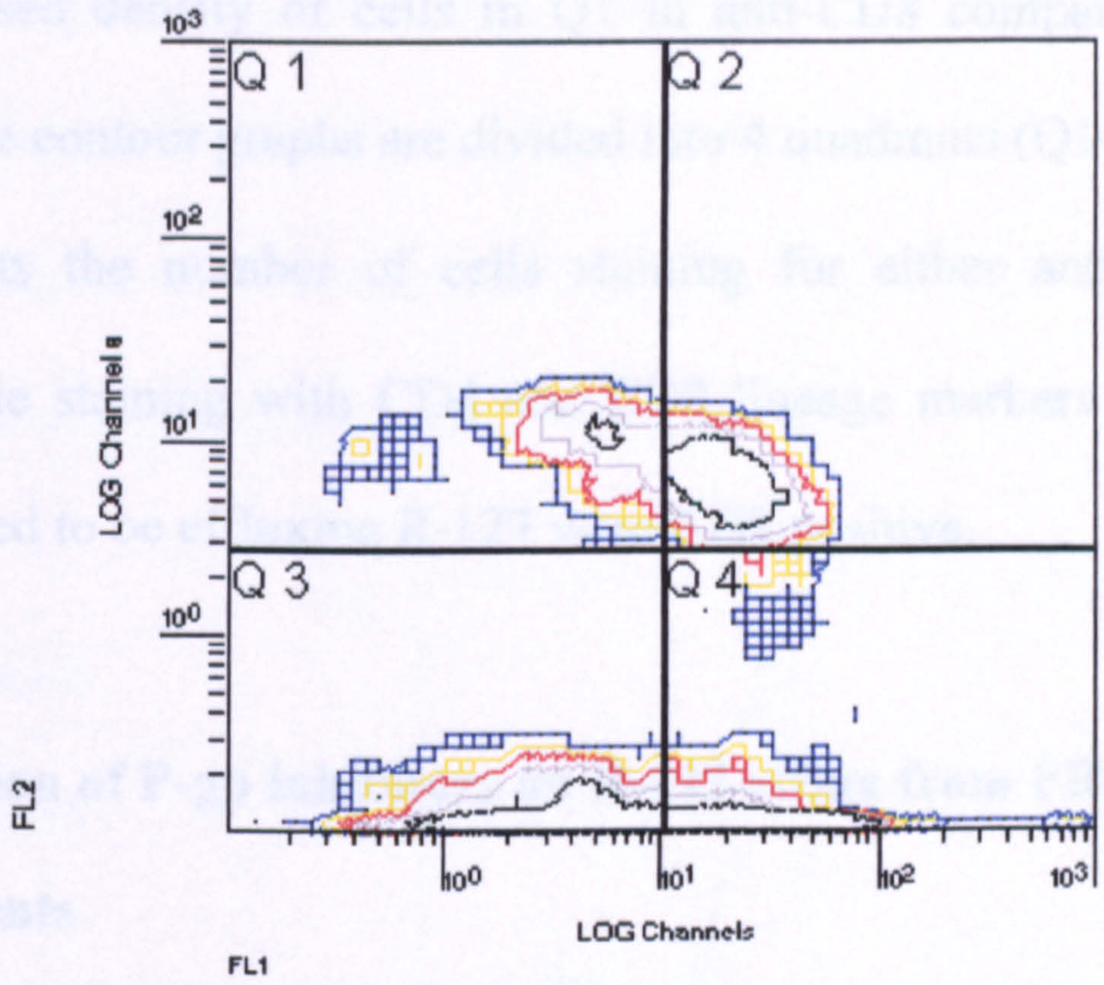
Introduction

T cells can be divided into 2 major classes, so called helper T cells and cytotoxic T cells. The antigens they express on the cell surface identify the classes of T cells. Helper T cells are characterised by expression of CD4 whereas cytotoxic T cells express CD8. Subsets of cells were assessed for ability to pump R-123.

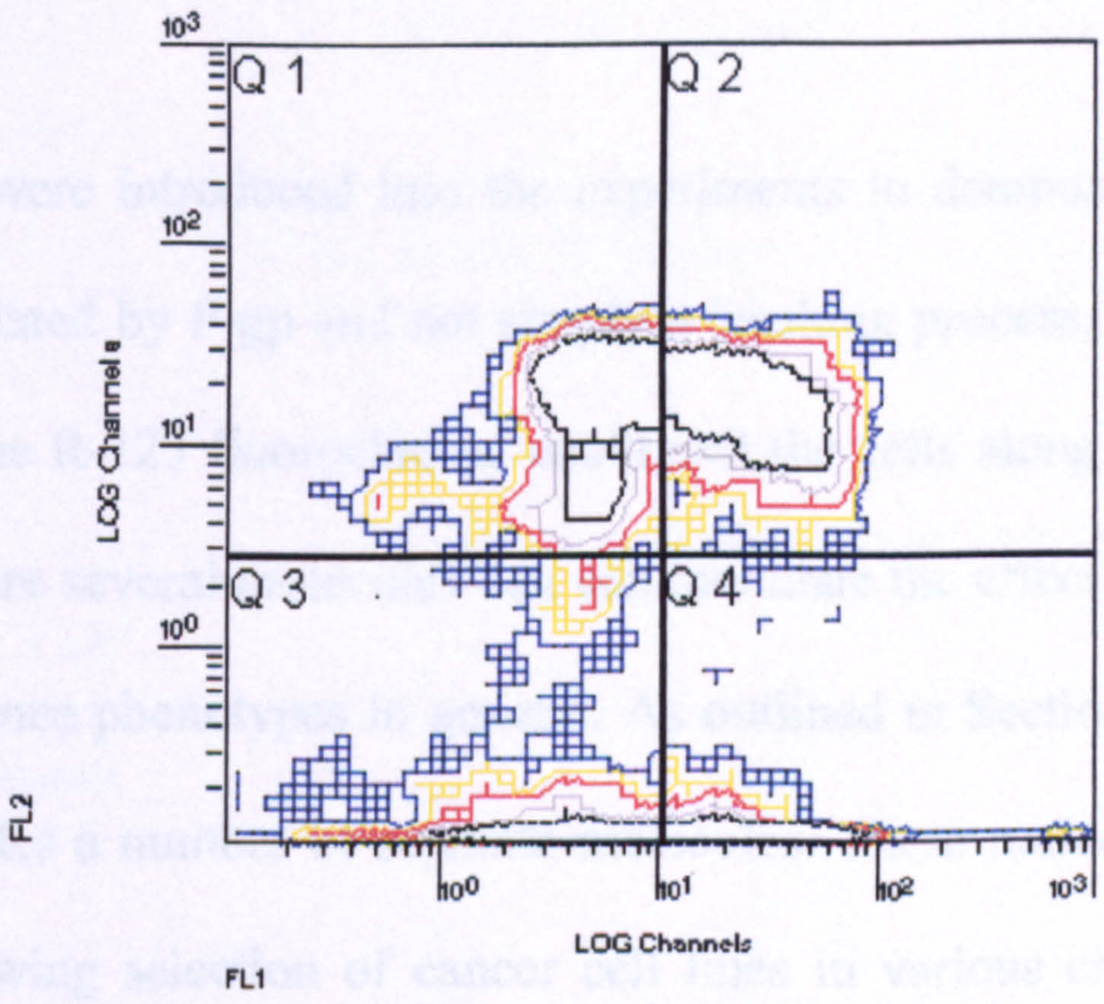
During these experiments cells were loaded with R123 and then incubated as before in R-123 free medium to allow efflux to occur. Following this the cells were harvested and counter stained with either CD8- or CD4-PE antibodies. The 2 colour staining was then analysed by flow cytometry.

Results

The results of experiments performed on 4 patient samples confirm that CD8 positive T cells are represented predominantly in the effluxing group and CD4 positive cells are represented in the R-123 retaining group as shown in Figure 5.5A and B.



(a)



(b)

Figure 5.5 Contour graph showing double staining of R-123 pumping cells ('x'-axis; FL1) with anti CD4 (a) or anti CD8 (b)-PE ('y'-axis; FL2). PBMC from renal transplant patients were loaded with 2μM R-123 for 30 minutes and subsequently incubated in R-123-free medium for a further 4 hours. The cells were harvested and counter-stained with either anti-CD4 or anti-CD8 monoclonal antibodies prior to FACS analysis. The areas with the greatest number of stained cells are represented by the inner black lines. The cell populations are subsequently less dense and scatterings of cells are represented in blue.

Note the increased density of cells in Q1 in anti-CD8 compared to anti-CD4 stained cells. The contour graphs are divided into 4 quadrants (Q1-4). The 'y'-axis (FL-2) represents the number of cells staining for either anti-CD4 or CD8. Following double staining with CD4 and CD8 lineage markers the majority of cells that appeared to be effluxing R-123 were CD8 positive.

5.2.3 Investigation of P-gp inhibitors on R-123 efflux from PBMC from renal transplant patients

Introduction

P-gp inhibitors were introduced into the experiments to demonstrate that efflux was indeed mediated by P-gp and not simply a leaching process, whereby over a period of time the R-123 fluorochrome could exit the cells along a concentration gradient. There are several molecules that can modulate the effect of P-gp and the multidrug resistance phenotypes in general. As outlined in Section 1.6, the MDR phenotype includes a number of separate molecules. These molecules have been discovered following selection of cancer cell lines in various chemotherapeutic drug solutions. The main molecules involved include P-gp, MRP and LRP. The hypothesis that the P-gp moiety would be most relevant to the study population described in this thesis was investigated. The expression and functional activity of P-gp can be studied using specific monoclonal antibodies and R-123 efflux respectively. There are also selective inhibitors of P-gp available including the cyclosporin analogue, PSC 833.

Preliminary experiments titrating PSC 833 at varying concentrations were performed (See Figure 5.6). The final concentration of PSC 833 in the cell cultures ranged from 1-20 μM. This experiment demonstrated that PSC 833 could inhibit R-123 efflux at concentrations as low as 1-2 μM. Therefore PSC 833 was added to subsequent experimental systems at 2 μM.

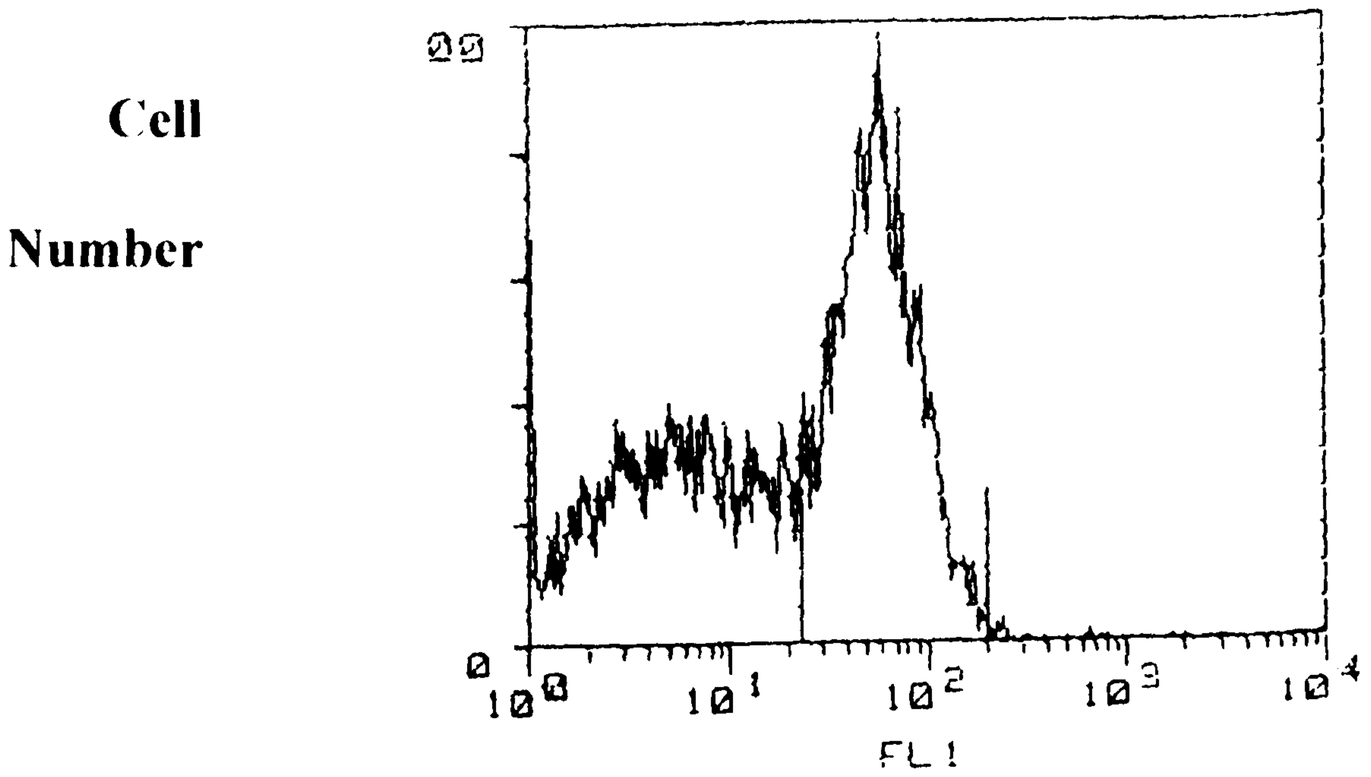


Figure 5.6 Effect of PSC 833 on inhibition of R-123 efflux. Cells from a single patient were loaded with 2μM R-123 and subsequently incubated in the absence of R-123 for up to 6 hours, in the absence (**a**) or presence of 2μM PSC 833 (**b**). Samples were kept on ice until FACS analysis.

Experiments to confirm this finding were performed on a further three patients and summarised in Table 5.5.

Pt	Time point	MFI for left side of graph	% cells effluxing R-123	MFI for right side of graph	% cells retaining R-123
	0 hr	0	0	143.9	100
A	6 hrs	10	49	78	51
	6 +PSC	0	0	112	100
	0 hr	0	0	98	100
B	6 hrs	33	53	140	47
	6+PSC	0	0	117.3	100
	0 hr	0	0	76	100
C	6 hrs	8	25	73	75
	6+PSC	0	0	99.9	100

Table 5.5 Effect of 2 μM PSC 833 on R-123 efflux in three patients. Cells from each patient were loaded with 2μM R-123 and subsequently incubated in the absence of R-123 for up to 6 hours, in the presence or absence of 2μM PSC 833.

In each case the addition of PSC 833 rendered the cells incapable of effluxing R-123. These results suggest that PSC 833 is indeed a powerful inhibitor of R-123 efflux. The solvent in which PSC 833 is dissolved as recommended by the manufacturer is ethanol/Tween 80 at 9:1 and then diluted in an equal volume of sterile distilled water (SDW). To examine any possible contaminant effect from the solvent the experiments were repeated comparing the effect of PSC 833 with that of solvent alone. The results are shown in Figure 5.7. This confirmed that the solvent could inhibit R-123 efflux sub maximally compared to PSC 833 in a dose dependent manner. Further control experiments were performed with ethanol

alone and sterile distilled water alone. Neither of these compounds affected R-123 efflux (Figure 5.8). The results of these experiments are summarised in Table 5.6.

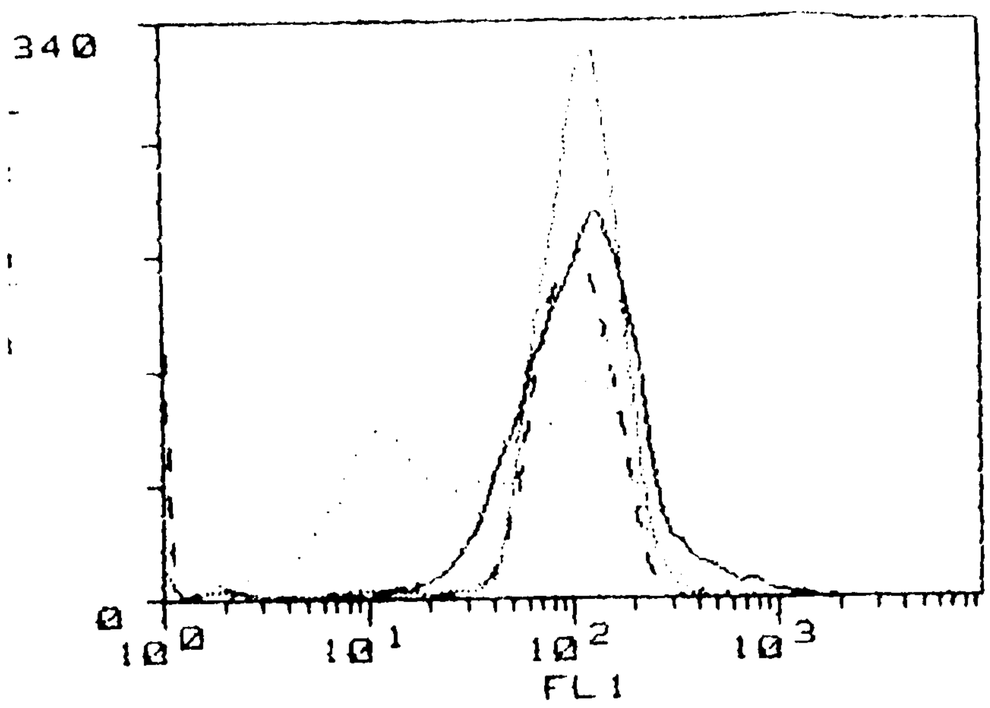


Figure 5.7 Comparison of PSC 833 and solvent on inhibition of R-123 efflux. Cells were loaded with 2 μM R-123 (continuous dark line) for 30 minutes and subsequently incubated in the absence of R-123 for up to 6 hours, in the presence (continuous light line) or absence (dotted line) of 2μM PSC 833 or solvent alone (dashed line). The cell samples were kept on ice until FACS analysis.

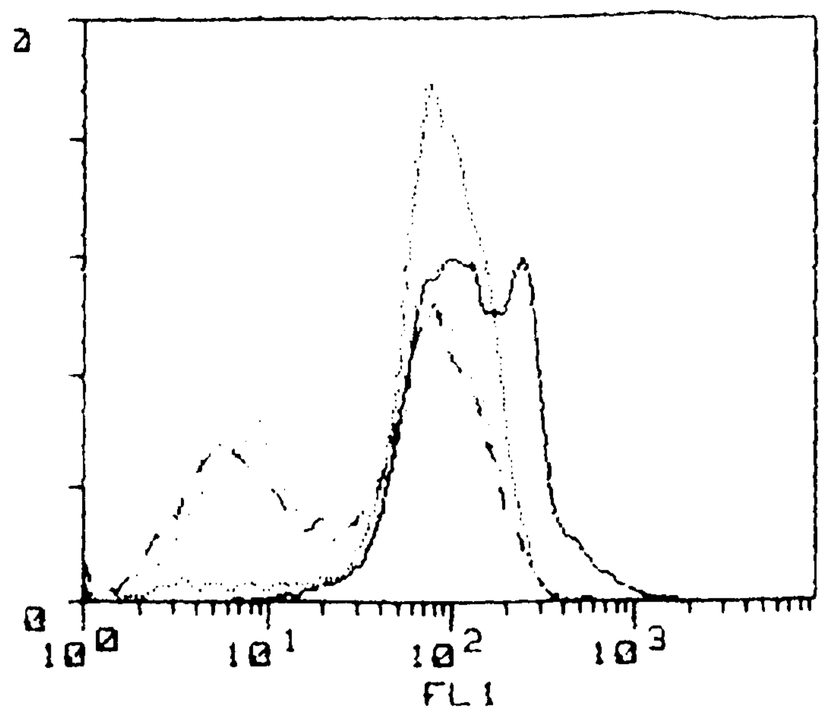


Figure 5.8 Comparison of PSC 833, ethanol and SDW on inhibition of R-123 efflux. Cells were loaded with 2μM R-123 for 30 minutes (continuous dark line) and subsequently incubated in the absence of R-123 for up to 6 hours, in the presence (continuous light line) or absence of 2μM PSC 833 with ethanol (dotted line) or sterile distilled water (dashed line) alone. The cell samples were kept on ice until FACS analysis.

Time point and drug	MFI 'left'	% cells effluxing R-123	MFI 'right'	% cells retaining R-123
0 hr	0	0	170	100
6 hr	8	37	98	63
PSC 833/6 hrs	0	0	114	100
Ethanol/6 hrs	6	38	82.1	62
SDW/6 hrs	7	35	92	65

Table 5.6 Effect of PSC 833, ethanol and SDW on R-123 efflux. R-123 efflux is shown to occur in the presence of ethanol and sterile distilled water. The addition of PSC dissolved in ethanol: SDW (1:1) renders the cells incapable of efflux.

Neither ethanol nor SDW have any effect on R-123 efflux. All experiments with PSC 833 were subsequently performed with ethanol: SDW as solvent.

The cell line MCF7/ADR was tested for R-123 efflux and inhibition by PSC 833. The results are shown in Figure 5.9.

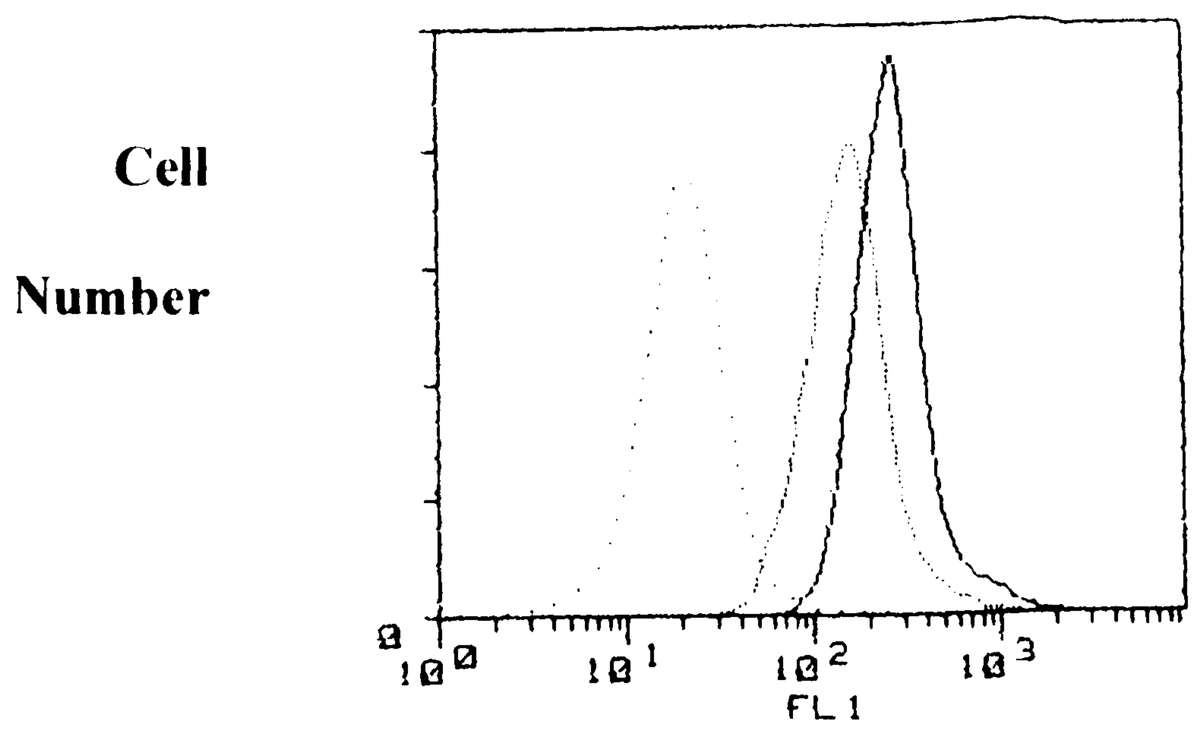


Figure 5.9 Effect of PSC 833 on R-123 efflux and inhibition in MCF7/ADR cells. The MCF7 cells were loaded with 2μM R-123 (continuous dark line) for 30 minutes and subsequently incubated in the absence of R-123 for 90 minutes, in the presence (continuous light line) or absence (dotted line) of 2μM PSC 833. The cells were kept on ice until FACS analysis.

The MCF7/ADR cells demonstrate a high level R-123 efflux that is not fully inhibited by 2 μM PSC 833. It is interesting to note that even in the loading sample, time 0 hour, there is a degree of pumping occurring that is not present when the cells are loaded with R-123 in the presence of PSC 833. These cells were used as positive control cells in R-123 pumping experiments and P-gp expression experiments.

5.2.4 Expression of P-gp by PBMC from renal transplant patients

Introduction

The experiments described in section 5.2.3 investigated drug pumping as exhibited by R123 efflux and inhibition with the selective P-gp inhibitor, PSC 833. The following experiments describe the expression of P-gp on PBMC. These experiments require the use of specific monoclonal antibodies (Mab) to P-gp. The Mab to external epitopes of P-gp, namely MRK16 and 4E3 were used. MRK16 and 4E3 are mouse anti-human P-gp monoclonal antibodies. The cell line MCF-7/ADR served as the positive control. A mouse IgG2a isotype-matched negative control antibody, diluted to the same concentration as the test antibody, was included in each experiment.

Control experiments

Each Mab was tested for binding to the positive control cell line MCF7/ADR and for isotype controls. The results are shown in Figure 5.10.

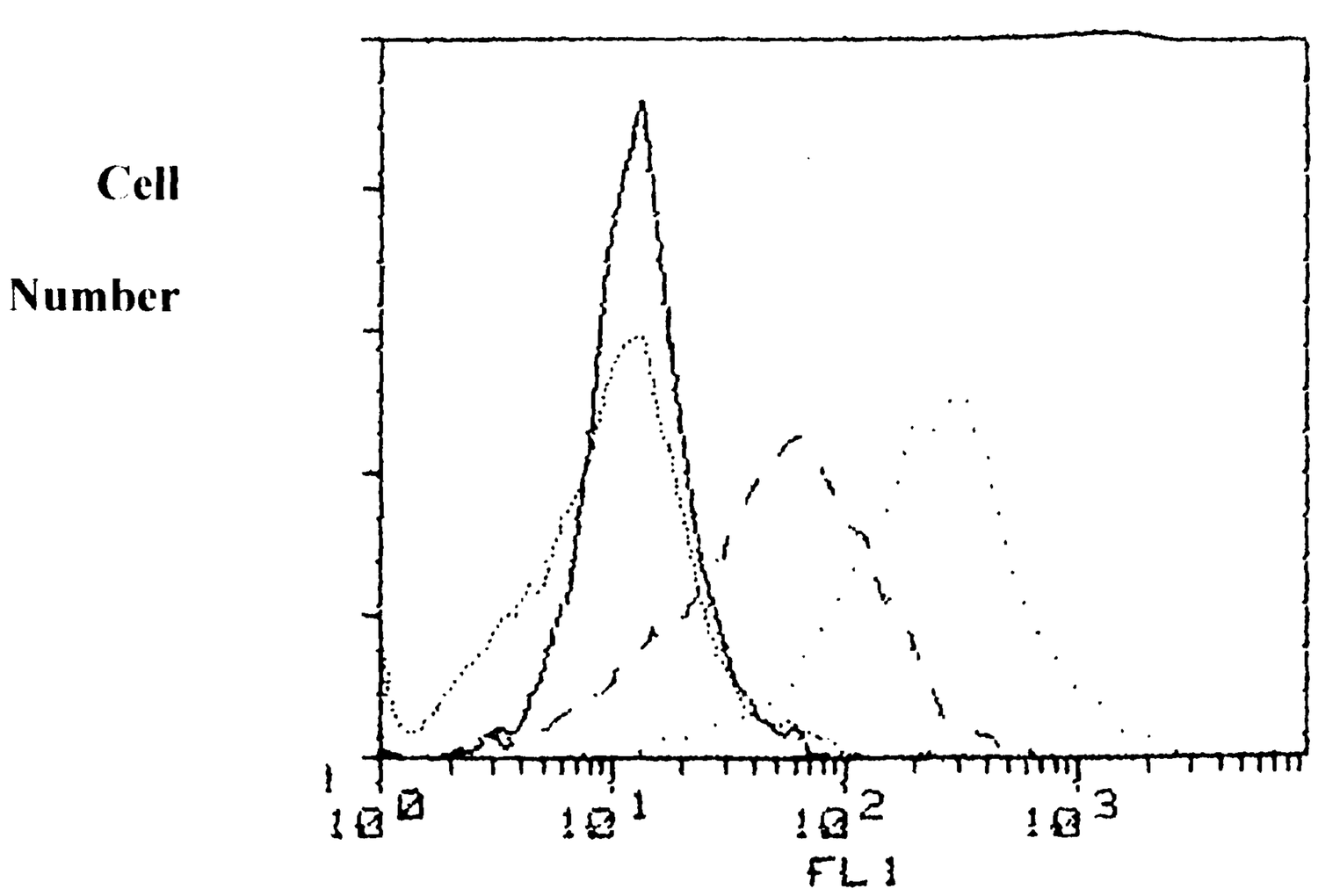


Figure 5.10 Expression of P-gp on the cell line MCF7/ADR following binding of MRK16 and 4E3. MCF7 cells were incubated with either MRK16 (dotted line) or 4E3 (dashed line) monoclonal antibodies as per manufacturers instructions. Isotype control antibodies (continuous lines) were established at the same concentration of antibody as with MRK16 and 4E3.

The histogram confirms high levels of staining with 4E3-FITC, and MRK16 added at 1μg and 2.5μg/assay. The isotype control antibody revealed low levels of background staining. These control experiments suggest that the Mab are working well, and can readily detect P-gp expressed on the surface of individual cells.

Expression of P-gp by PBMC from renal transplant patients

The positive control cell line worked well as before. However, MRK16 staining on PBMC showed only marginal increase in MFI over isotype control. The MFI of isotype alone was 3.4 and MFI of MRK16 stained cells was 4.6 (See Figure 5.11).

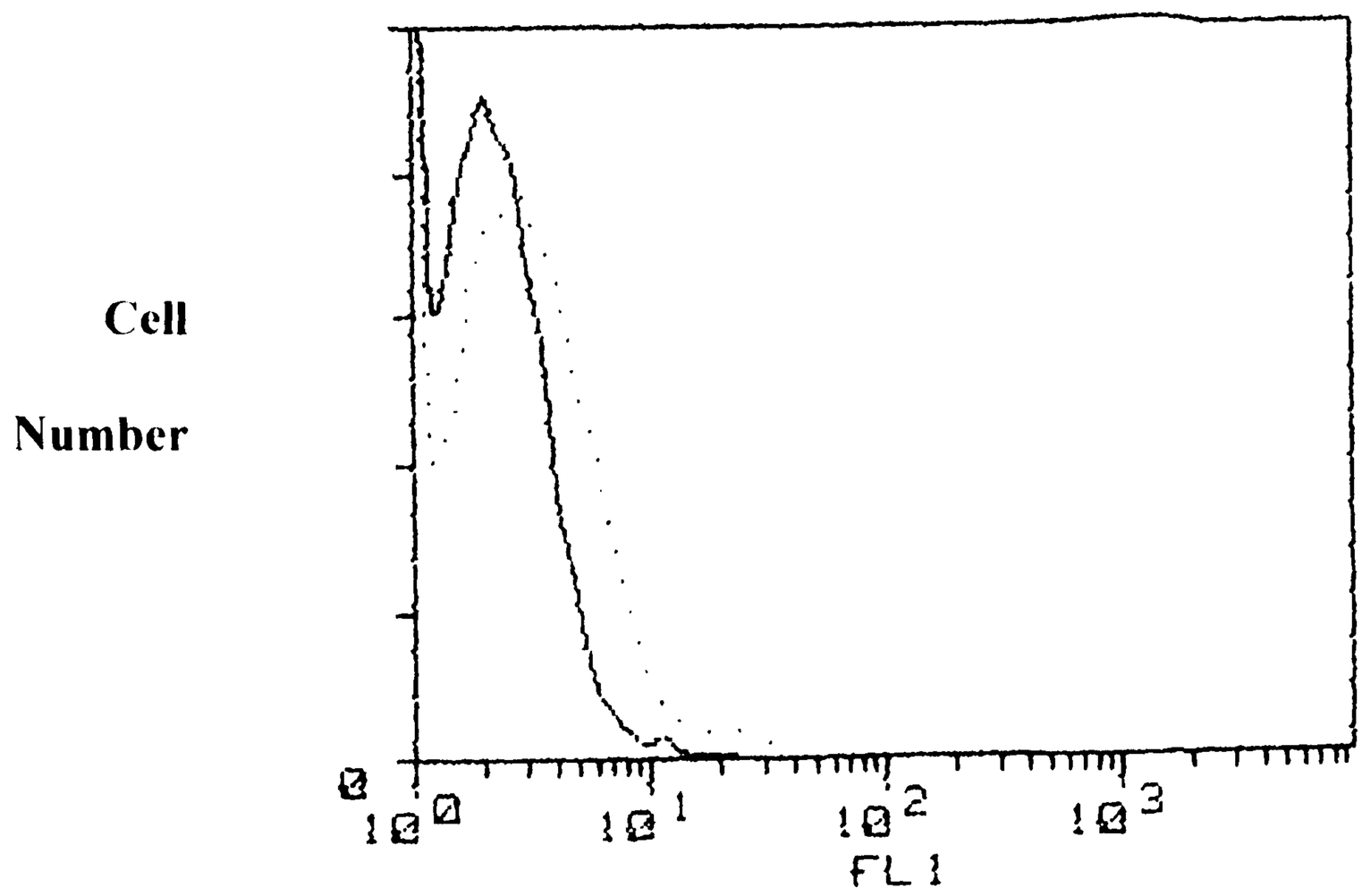


Figure 5.11 Staining of PBMC with MRK16 Monoclonal antibody. PBMC from a single patient were treated with 2.5µg of MRK16 (dotted line) and isotype control (continuous line) as per manufacturers instructions.

It was clear during the running of the samples through the flow cytometer that there was a high level of non-specific binding of the isotype control antibody to the larger monocyte population of cells. This was reduced in subsequent analysis by manually gating on the lymphocyte population. Further patient samples yielded

similar result with negligible binding of MRK16 or 4E3 Mab and are presented in Table 5.7.

Patient No.	Test Monoclonal Antibody	Mean Fluorescent Intensity of Isotype Control Antibody	Mean Fluorescent Intensity of Test antibody
22	4 E 3	5	11.5
22	MRK16	3.4	4.6
18	MRK16	1.4	2.2
21	MRK16	1.6	1.36
21	MRK16	6.52	7.76
7	MRK16	7.1	8.4
4	MRK16	7.07	11.81
4	4 E 3	8.74	8.62
2	MRK16	1.35	2
7	MRK16	3.96	9.9
	MRK16	18.6	16.4
14	MRK16	1.41	1.54
	MRK16	9.12	15.6
3	MRK16	6.56	10.39
30	MRK16	6.79	
22	MRK16	7	18.9
29	MRK16	2.5	3.26
32	MRK16	2.06	2.43
38	MRK16	1.42	1.88
19	MRK16	2.61	5.45
26	MRK16	4.25	8.06
MCF-7/ADR:	MRK16	**3.15**	**190.3**

Table 5.7 Summary of antibody staining for P-gp expression in PBMC from renal transplant patients. PBMC were treated with both MRK16 and 4E3 monoclonal antibodies. Each assay included an isotype matched control antibody. There is negligible staining of the PBMC with MRK16 and 4E3 above the isotype control. The positive control cell line stained very strongly following treatment with MRK16.

Since the patients' PBMC tested were uniformly negative for P-gp expression a number manipulations in the experimental design were investigated. For example,

pre-fixing the cells in 2% formaldehyde and 100% acetone; use of other secondary antibody conjugates [phycoerythrin (-PE)]; use of the primary antibody at higher concentration per assay and the use of fresh rather than cryopreserved cells. These manipulations did not affect the outcome and only the results with freshly isolated PBMC will be presented.

Experiments with freshly isolated cells

Freshly isolated PBMC from 4 patients were tested for P-gp expression using 4E3-FITC and MRK16 Mab.

Results

MCF7/ADR positive control cell line		
	Isotype control: MFI	12.05
	4E3-FITC	339.27
	MRK16	837.59
Mean of 4 patients	Isotype control	2.45 +0.23
	4E3-FITC	3.20 +1.4
	MRK16	5.68 +2.16

The data suggest no demonstrable binding of the monoclonal antibodies to PBMC membranes.

Conclusion

These studies show that the expression of P-gp on PBMC membranes was not demonstrable. The monoclonal antibodies tested bound to the positive control cells well and there was little non-specific binding. This result is paradoxical in view of the data obtained with R-123. The possible reasons will be discussed in Section 6. 2.

5.2.5 Effect of P-gp inhibition on the anti-proliferative effects of Cy A and FK506

Introduction

Since lymphocytes have drug-pumping mechanisms that are inhibited by selective inhibitors of P-gp could inhibition of drug pumping influence the sensitivity of PBMC to immunosuppressant drugs? The effect of the selective P-gp inhibitor, PSC 833, on ID_{50}s for the two drugs was determined.

PBMC cultures were established as described in Section 4.2.4, and dose response curves obtained following the addition of graded doses of Cy A and FK506. Similar cultures were established following the addition of varying doses of the P-gp inhibitor PSC 833. These cultures were established to observe any effect on cell proliferation from abrogating the ability of the cells to expel immunosuppressant drugs from the interior of cells to the exterior medium. If the ability of the cell to expel these drugs by MDR1-associated mechanisms is crucial to the sensitivity of cells to the drugs then the sensitivity of cells to the anti-proliferative effects of Cy A and FK506 would be enhanced following P-gp inhibition.

Results

Dose response curves were obtained for the effect of Cy A alone and in combination with PSC 833 and an example is shown in Figure 5.12.

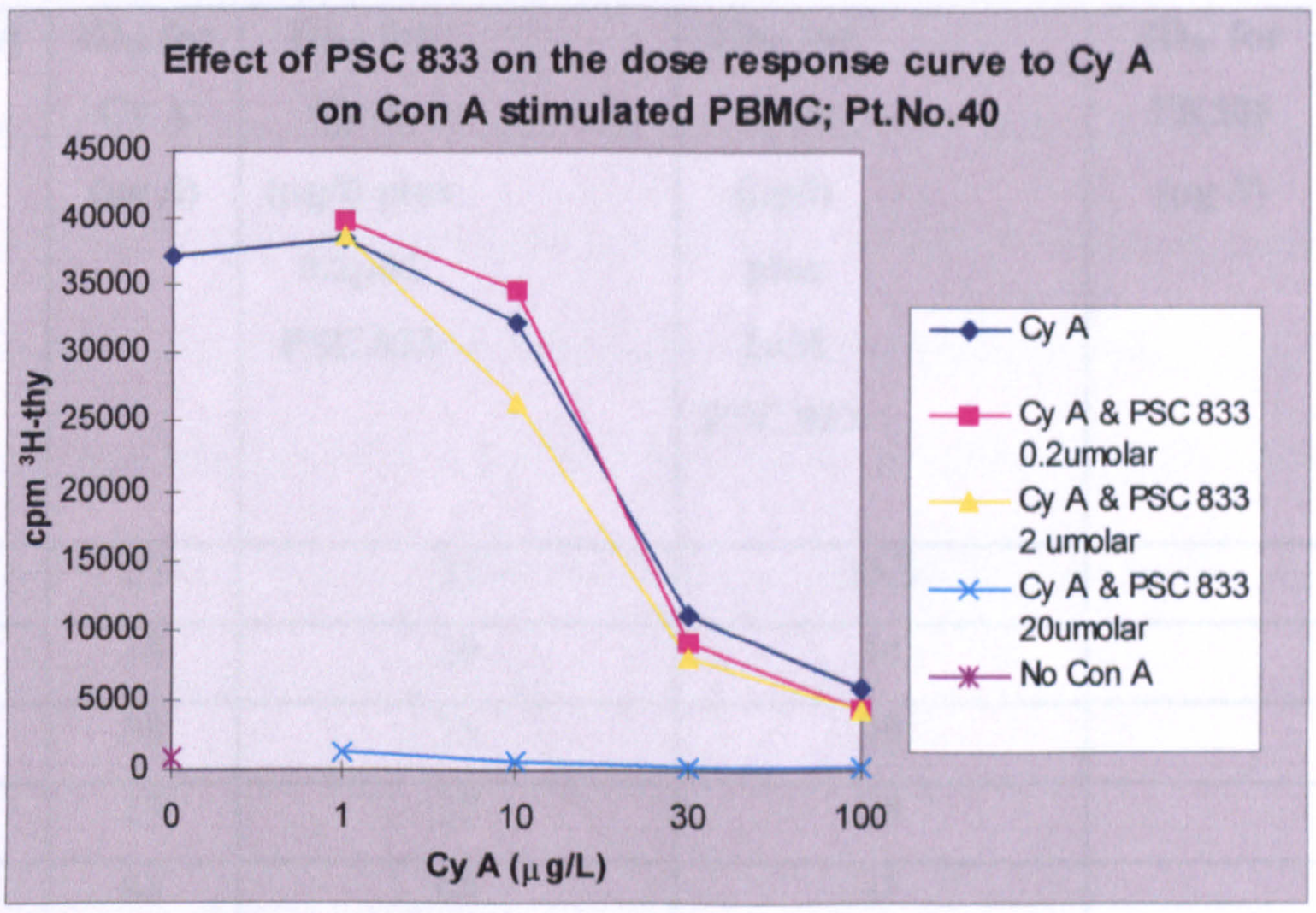


Figure 5.12 Effect of PSC 833 on the proliferative response of Con A stimulated PBMC treated with Cy A. PBMC from a single patient were incubated in the presence or absence of PSC 833 and Cy A, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations.

The data points are the mean of two values. From this graph the ID_{50}s can be established for Cy A alone and Cy A plus PSC 833 at varying concentrations. The graph demonstrates that Cy A inhibits PBMC proliferation following mitogenic stimulation by Con A in a dose dependent manner. The graph also shows that Cy A inhibits PBMC proliferation in the presence of PSC 833 at concentrations of 0.2μM and 2μM. At a concentration of 20μM, PSC 833 obliterates any proliferation of PBMC after mitogenic stimulation regardless of dose of Cy A.

The impact of PSC 833 at the lower concentration 0.2μM is negligible. However, there appears to be a shift in the dose response curve to the left when PSC 833 is added at 2μM. Similarly, dose response curves were obtained in the presence of FK506 with or without addition of PSC 833. Table 5.8 is a summary of the ID_{50}s obtained for all patients studied.

Patient No.	ID_{50} for Cy A (μg /l)	ID_{50} for Cy A (μg/l) plus 0.2μM PSC 833	ID_{50} for Cy A (μg/l) plus 2μM PSC 833	ID_{50} for FK506 (μg /l)	ID_{50} for FK50 (μg /l) plus PSC 833 2μM
39	25	21	15.5		
40	18	20	14		
7	38	25	20		
41	23	27	28		
42	68	64	47		
43	16	15	20		
37	48	32	23		
44	58		78		
45	21	18	15		
46				0.044	0.04
47				0.058	0.05
48				0.12	0.17
29				1.2	1.2
49				1.7	1.4
Mean	**35**	**27.8**	**28.9**	**0.62**	**0.57**
SD	**19**	**15.6**	**21**	**0.77**	**0.67**

Table 5.8 Effect of addition of PSC 833 to ID_{50}s for Cy A and FK506. Dose response curves were obtained for each patient to Cy A or FK506 and compared to the curves obtained in the presence of the selective P-gp inhibitor, PSC 833. Paired t-Test for Cy A: Cy A/PSC 833 (2μM) p=0.24;Paired t-Test for FK506 : FK506/PSC 833 p 0.45.

The results in Table 5.8 demonstrate that PSC 833 did not significantly affect the ID_{50s} in either Cy A or FK506 treated cells. The mean ID_{50} for Cy A alone was 35 µg/l (SD 19 range 16-68) and the mean ID_{50} for Cy A plus PSC 833 at 2µM was 28.9 µg/l (SD 20.9 range 15-78). This was not statistically significant as tested by the paired t-Test (p= 0.24). The mean ID_{50} for FK506 alone was 0.62 µg/l (SD 0.77 range 0.044-1.75) and the mean ID_{50} for FK506 plus PSC 833 was 0.57 µg/l (SD 0.67 range 0.04-1.4: p =0.45). The concentration of PSC 833 used (2µM) is equivalent to the concentration used to inhibit R-123 efflux. This concentration in molar terms is approximately 8 fold higher than the highest dose of Cy A used. Similarly, the addition of 0.2µM PSC 833 causes a reduction in the mean ID_{50} compared to Cy A alone but again this does not reach statistical significance when subjected to a paired t-test (p=0.12).

Experiments to assess any direct anti-proliferative effect of PSC 833

Since PSC 833 is a cyclosporin analogue any direct effect on the proliferative response of PBMC was examined. Titration experiments of PSC 833 at doses of 0.2, 2 and 20 µM were performed in the absence of Cy A. The results or these experiments are shown in Table 5.9.

Patient No.	ID_{50} for Cy A (µg /l)	Mean cpm Con A only	Mean cpm Con A/PSC 833 0.2µM	Mean cpm Con A/PSC 833 0.6µM	Mean cpm Con A/PSC 833 2µM	Mean cpm Con A/PSC 833 6µM	Mean cpm Con A/PSC 833 20µM
7	38	64124	61717.5		56634.5		1108.5
41	23	28678.7	27696.7		29081.4		401.65
42	68	46904.85	45536.4	56772.2			
43	16	37009.85	38170.7	33748.5	33957.9		
37 a	48	16286.9	17832.6	19089.3	23523		
44	58						
46	21	26339	24968	34174.6	23161.2		
51		63215.7	66556.3	65818.1	61996.3	37476.7	
52		84139.9	93230.5	79253.5	82238.4	49002.2	
37 b		31242.6	31136.5	29136.2	25555.7	5291.6	
Mean		**44215.3**	**45205**	**45427.5**	**42018.6**		
SD		**22135.4**	**24361.4**	**22025.8**	**22140**		

Table 5.9 Effect of PSC 833 on Con A stimulated PBMC. Cell cultures were established following stimulation with 5µg /ml Con A. The effect of PSC 833 on cell proliferation was explored, and the counts per minute (cpm) recorded.
Paired t-Test comparing no PSC 833:0.2µM PSC 833: p 0.42
Paired t-Test comparing no PSC 833:0.6µM PSC 833: p 0.41
Paired t-Test comparing no PSC 833:2.0µM PSC 833: p 0.27

It can be seen from the Table 5.9 that there is an anti-proliferative effect of PSC 833 at the high concentrations of 6 and 20µM. At 2µM there is variation in response with some samples showing a direct anti-proliferative effect (7, 43,44b) and the other samples demonstrating no appreciable effect. This is reflected in the means of the 2 groups and overall no statistical effect.

PSC 833 exhibited an effect on Con A stimulated cells at a concentration of 6µM. At 2µM a direct effect was variable and not significant. This is important since it

demonstrates that the P-gp inhibitor is not toxic at this dose. By inference, the effect of PSC 833 on R-123 efflux indicates that the retention of dye following addition of PSC 833 was not simply cell toxicity, but a pharmacological effect of the drug.

Summary of Sections 5.1-5.2.5

There is a dose dependent inhibition of lymphocyte proliferation following the addition of the immunosuppressive drugs Cy A and FK506. There is a wide variation in sensitivity to the drugs. The hypothesis that MDR1-associated mechanisms may be responsible for this variation remains unproved. However, it has been demonstrated that drug pumping occurs in all the patient samples as assessed by R-123 efflux and inhibition with PSC 833. This suggests that the pumping is due to P-gp functional activity. It has not been possible to demonstrate P-gp expression on the patients' samples of PBMC although the antibodies worked well with the positive control cell line MCF-7/ADR. The majority of cells pumping R-123 are CD8 positive T cells. PSC 833 did not significantly affect the ID_{50s} in either Cy A or FK506 treated PBMC when stimulated by Con A.

Conclusion

The data demonstrate that P-gp dependent mechanisms are not responsible for the variation in sensitivity of PBMC to the immunosuppressant drugs Cy A and FK506.

5.3 Investigation of the interaction of immunosuppressant drugs on PBMC from renal transplant patients

Introduction

As discussed in Section 1.5 the idea behind the work described in this thesis was to develop a means of studying *in vitro* patient lymphocyte responses to immunosuppressant drugs in order to understand their mode of action, mechanisms of resistance, and interactions between them. Another approach to optimising immunosuppressant drug regimes is to use multiple drug combinations at lower than conventional individual doses. Drug combinations that demonstrate synergy may improve efficacy and side effect profile. Recently, rapamycin has been introduced into clinical transplantation. Rapamycin and a synthetic analogue, SDZ RAD, have a different mechanism of action to FK506 but share the same intracellular binding site (see Section 1.3.4-1.3.6). The interaction of FK506 with SDZ RAD was explored in PBMC from renal transplant patients.

Lymphocyte cultures were established in two systems, namely Con A stimulated lymphocyte cultures and Mixed Lymphocyte Reactions (MLR). The MLR was employed for the reasons outlined in Section 3.1.3. These cultures were performed with graded doses of SDZ RAD alone and with SDZ RAD in combination with low dose FK506 (less than the mean ID_{50} in the previous work in Section 5.1). Lymphocyte proliferation was assessed in two ways. Firstly, following

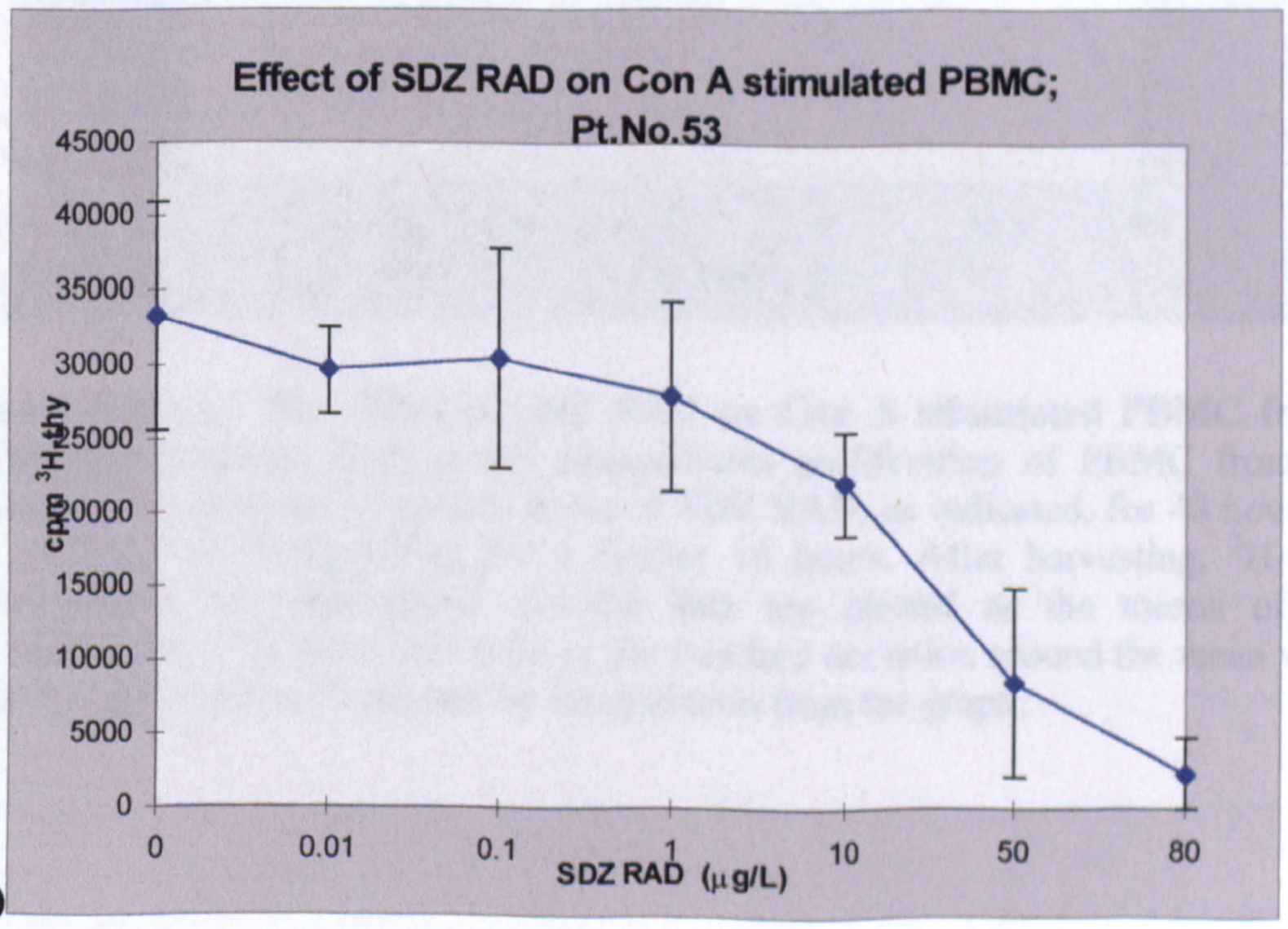
incorporation of ^{3}H-thymidine (^{3}H-thy) and secondly by expression of CD25 on the cell surface using flow cytometry.

5.3.1 Con A data

To assess the interactions between the drugs the effects of the drugs on a maximal stimulus provided by Con A were investigated. The experimental method has been described in Section 4.2.4. The results are divided into preliminary dose response curves for SDZ RAD and the effects of combining SDZ RAD with FK506. (Patient characteristics are presented in Table 4.1).

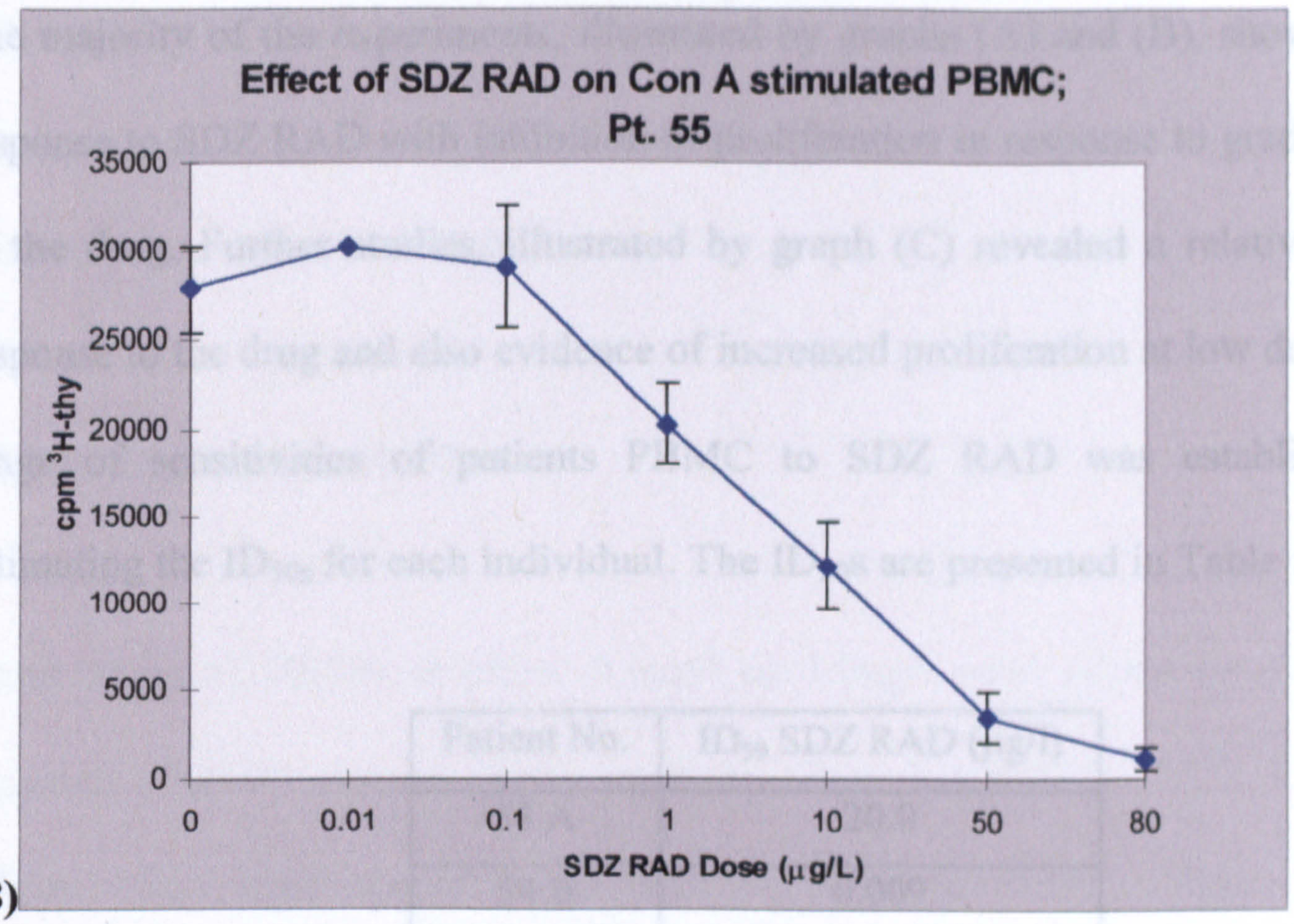
5.3.1.1 SDZ RAD dose response curves

The results are presented graphically to illustrate the dose response curves obtained with SDZ RAD (see Figure 5.13). The ID_{50}s obtained in the presence of SDZ RAD are presented in Table 5.10.



(A)

(B)



(C)

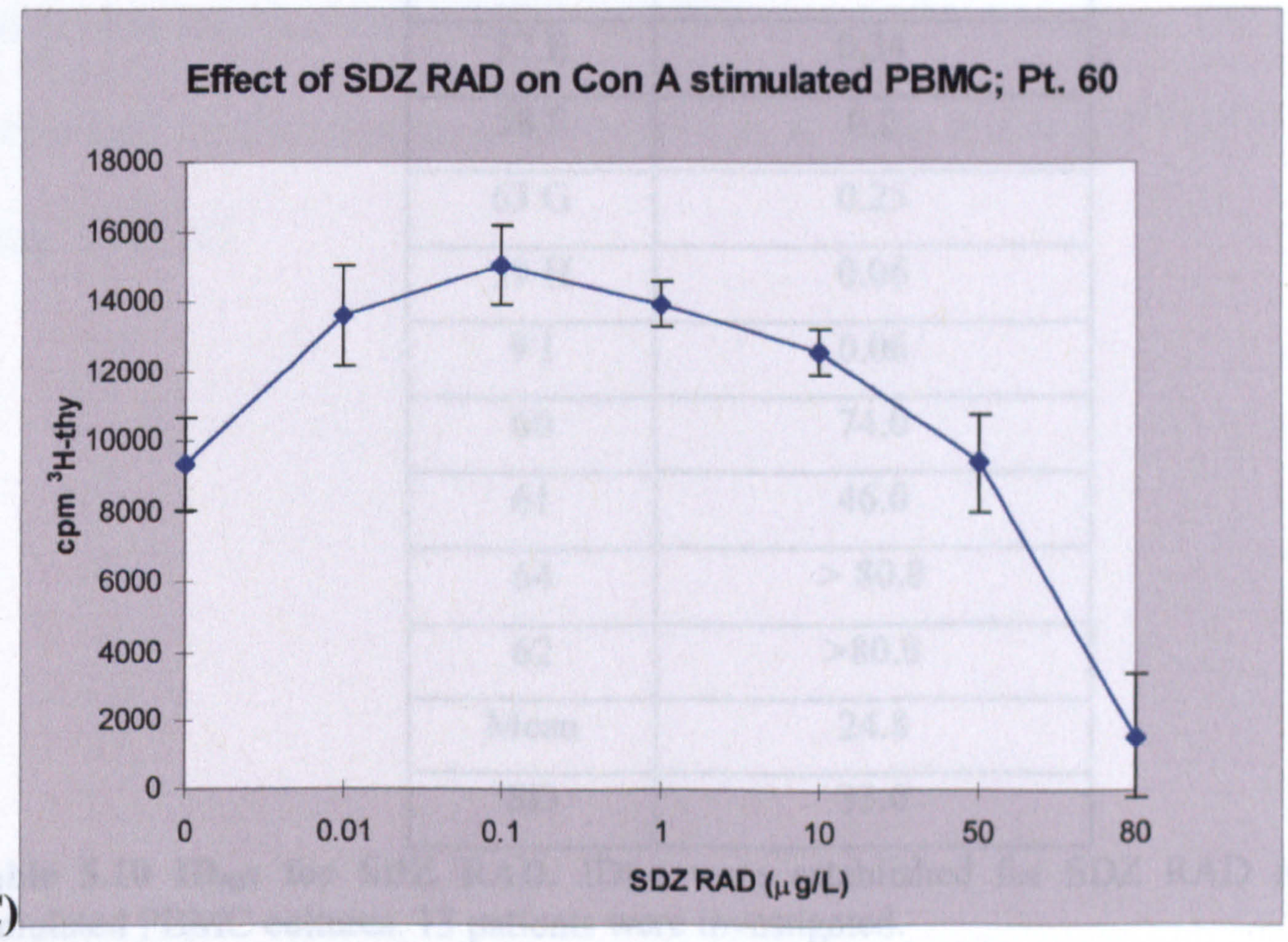


Figure 5.13A-C. The effect of SDZ RAD on Con A stimulated PBMC from renal transplant patients. Each graph demonstrates proliferation of PBMC from a single patient in the presence of graded doses of SDZ RAD, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as the means of replicate determinations. The error bars refer to the standard deviation around the mean value. The ID_{50} for SDZ RAD are obtained by interpolation from the graph.

The majority of the experiments, illustrated by graphs (A) and (B), show a good response to SDZ RAD with inhibition of proliferation in response to graded doses of the drug. Further studies, illustrated by graph (C) revealed a relatively poor response to the drug and also evidence of increased proliferation at low doses. The range of sensitivities of patients PBMC to SDZ RAD was established by estimating the ID_{50}s for each individual. The ID_{50}s are presented in Table 5.10.

Patient No.	ID_{50} SDZ RAD (µg/l)
53 A	20.0
54 B	0.009
55 C	5.1
56 D	16.0
57 E	0.34
58 F	0.2
63 G	0.25
59 H	0.06
91	0.06
60	74.0
61	46.0
64	>80.0
62	>80.0
Mean	24.8
SD	33.0

Table 5.10 ID_{50}s for SDZ RAD. ID_{50}s were established for SDZ RAD in Con A stimulated PBMC cultures. 13 patients were investigated.

The mean for the whole group is 24.8 µg/l (SD 33.0 range 0.009-80.0). Subgroup analysis of the 9 patients *A-I* confirms a mean ID_{50} for these 9 patients of 4.7µg/l (SD 7.8 range 0.009-20). From the data presented in Section 5.1, the mean ID_{50} for FK506 was 1.2 µg/l (SD 1.9 range 0.085-5.5). The ratio of the mean ID_{50}s for the drugs SDZ RAD and FK506 is 3.9. The other subset of patients behaves

differently. The mean ID_{50} for this subset is 70 µg/l (SD 16.2 range 46-80). Further experiments were performed to characterise the effect of FK506 on the dose-response curve to SDZ RAD.

5.3.1.2 SDZ RAD & FK506

Fixed doses of FK506 at either 0.1µg/l or 1.0µg/l were added to the Con A stimulated PBMC cultures in the presence of graded doses of SDZ RAD. The effects of SDZ RAD in combination with FK506 are presented in Figure 5.14A-H. Each patient is represented by two graphs. The first depicts the actual levels of cell proliferation as measured by incorporation of ^{3}H-thymidine. The second is normalised for the response obtained to Con A in the absence of SDZ RAD ('No Drug' Control).

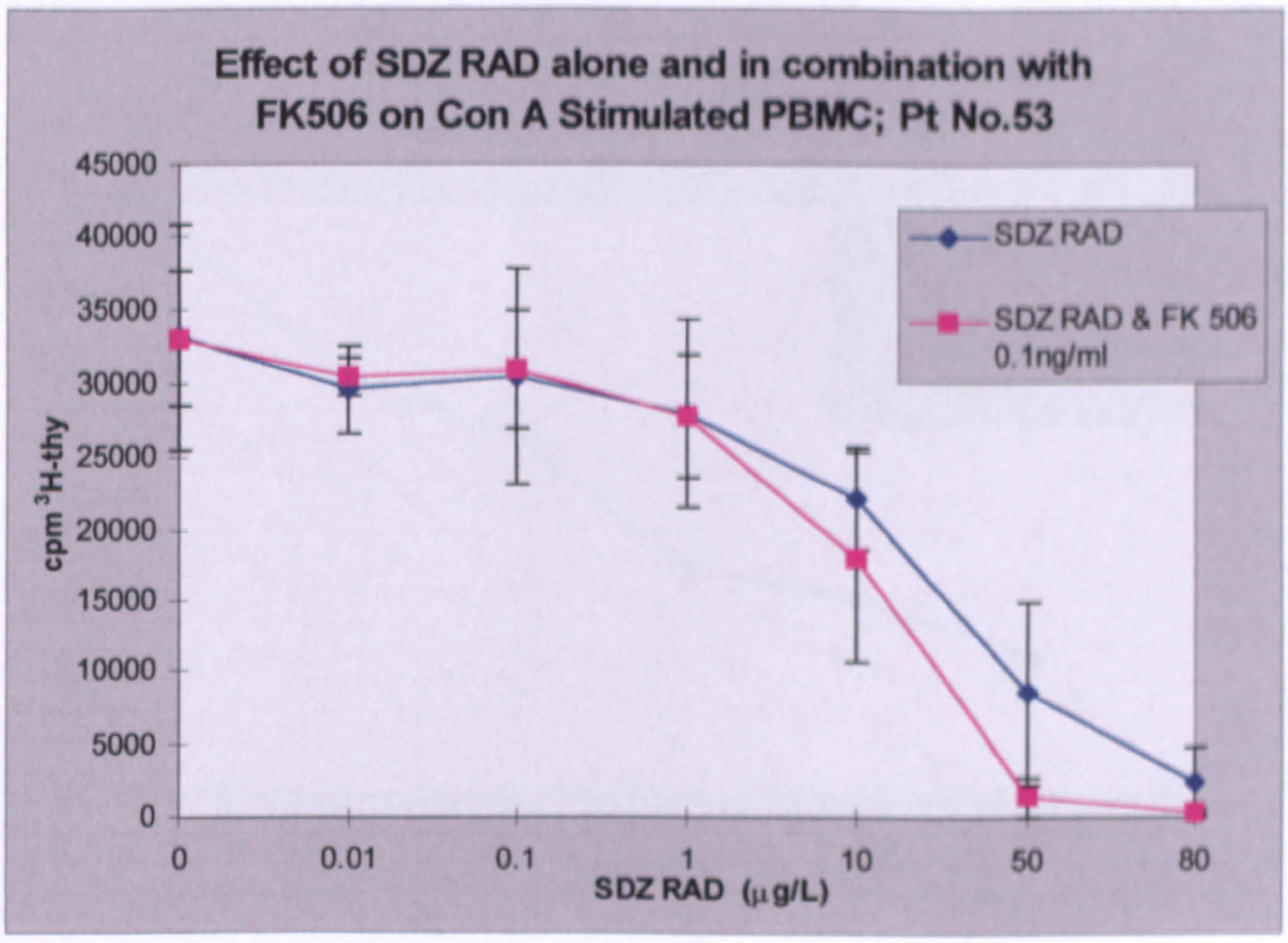


Figure 5.14A Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC. PBMC from a single patient were incubated in the presence or absence of SDZ RAD and FK506, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars refer to the standard deviation around the mean value. ID_{50}s were obtained for SDZ RAD alone, and for SDZ RAD in combination with FK506 by interpolation of the graphs.

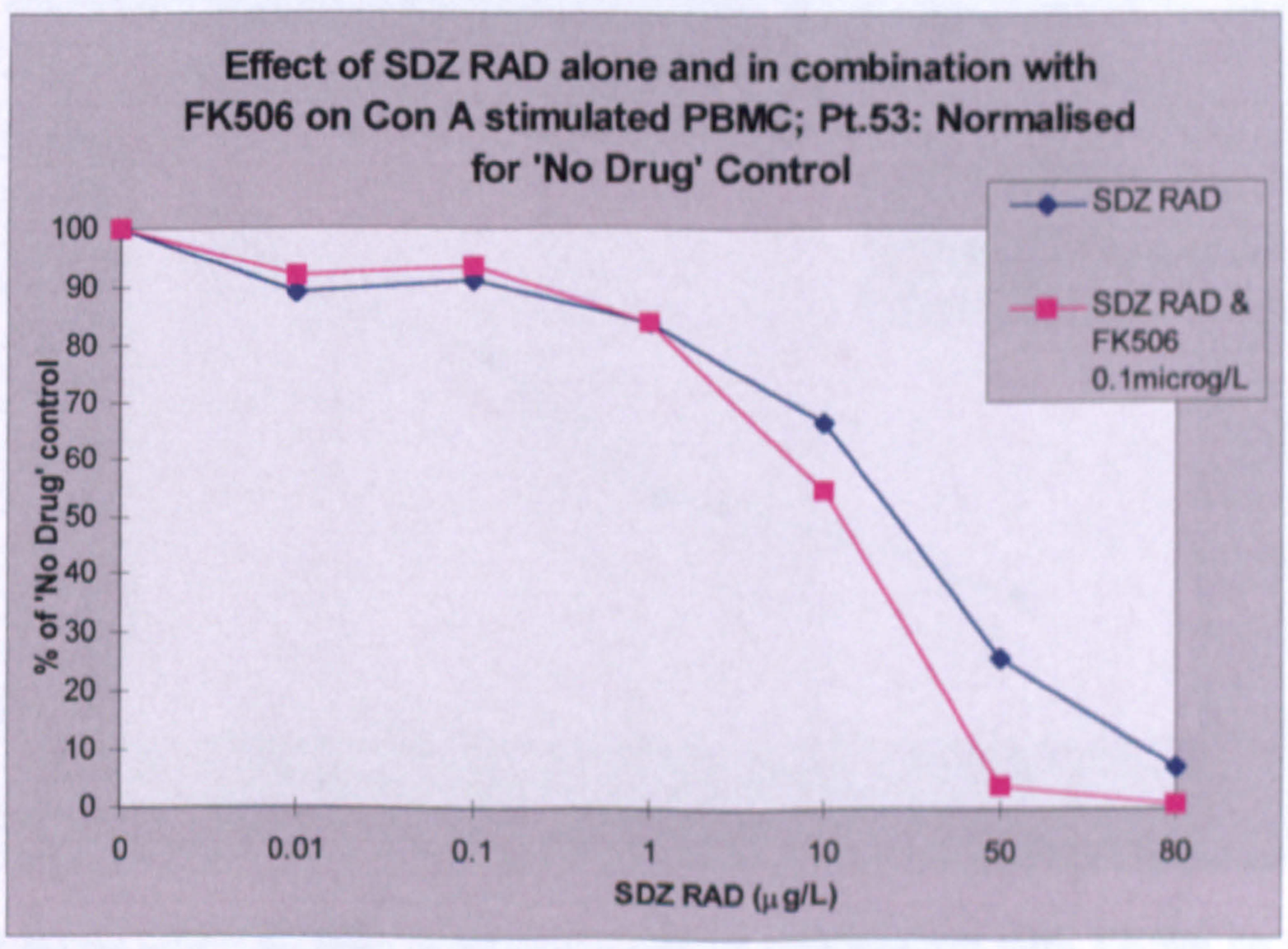


Figure 5.14B Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC; Pt. 53 Normalised for 'No Drug' control. The data were normalised by assigning '100%' to the level of proliferation obtained in the absence of any drug and calculating the percentage of inhibition achieved with respect to the 100% level of proliferation for both SDZ RAD alone and SDZ RAD in combination with FK506.

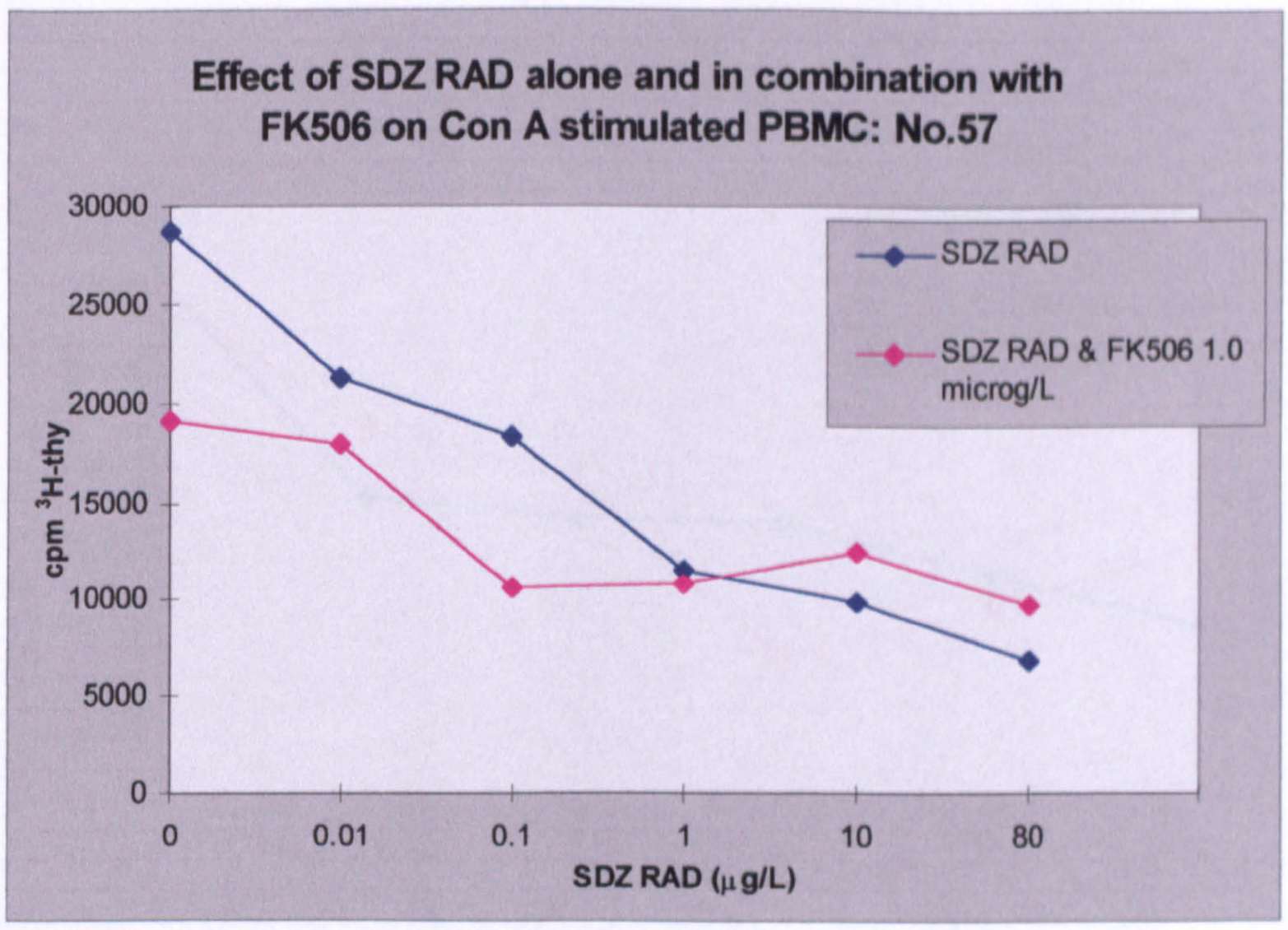


Figure 5.14C Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC. PBMC from a single patient were incubated in the presence or absence of SDZ RAD and FK506, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. ID_{50}s were obtained for SDZ RAD alone, and for SDZ RAD in combination with FK506 by interpolation of the graphs.

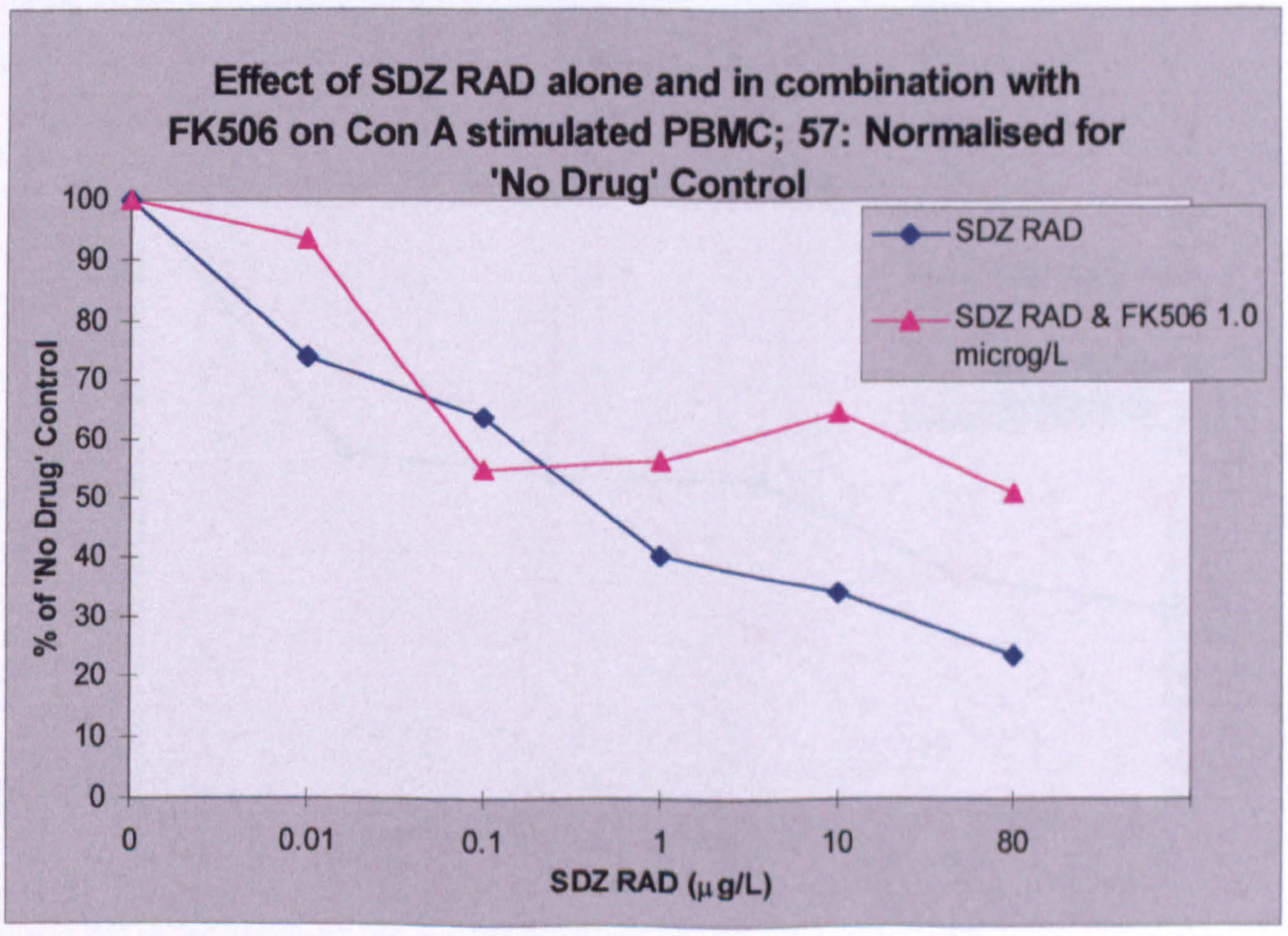


Figure 5.14D Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC; normalised for 'No Drug' control. The data were normalised by assigning '100%' to the level of proliferation obtained in the absence of any drug and calculating the percentage of inhibition achieved with respect to the 100% level of proliferation for both SDZ RAD alone and SDZ RAD in combination with FK506.

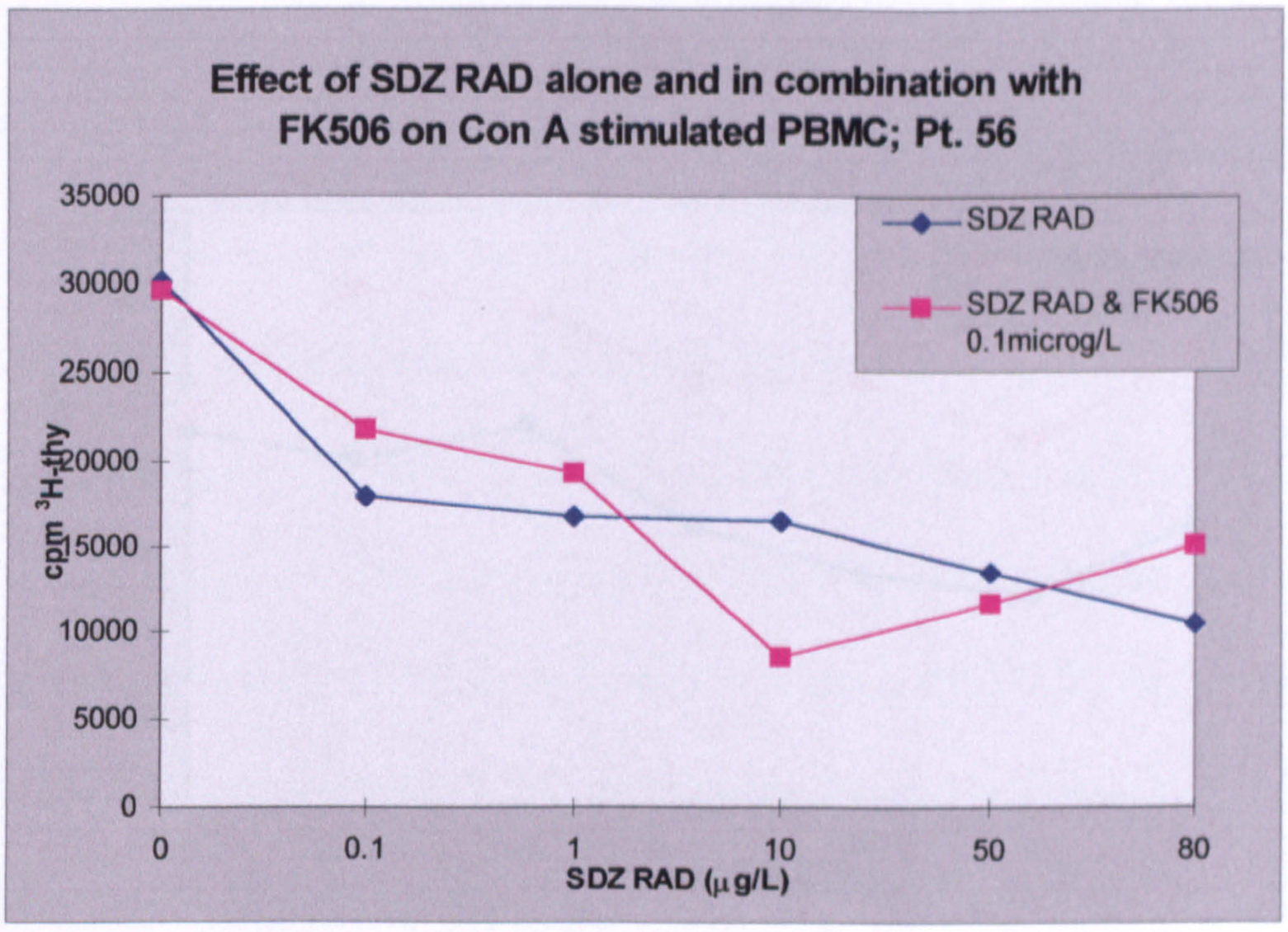


Figure 5.14E Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC; Pt. 56. PBMC from a single patient were incubated in the presence or absence of SDZ RAD and FK506, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. ID_{50}s were obtained for SDZ RAD alone, and for SDZ RAD in combination with FK506 by interpolation of the graphs.

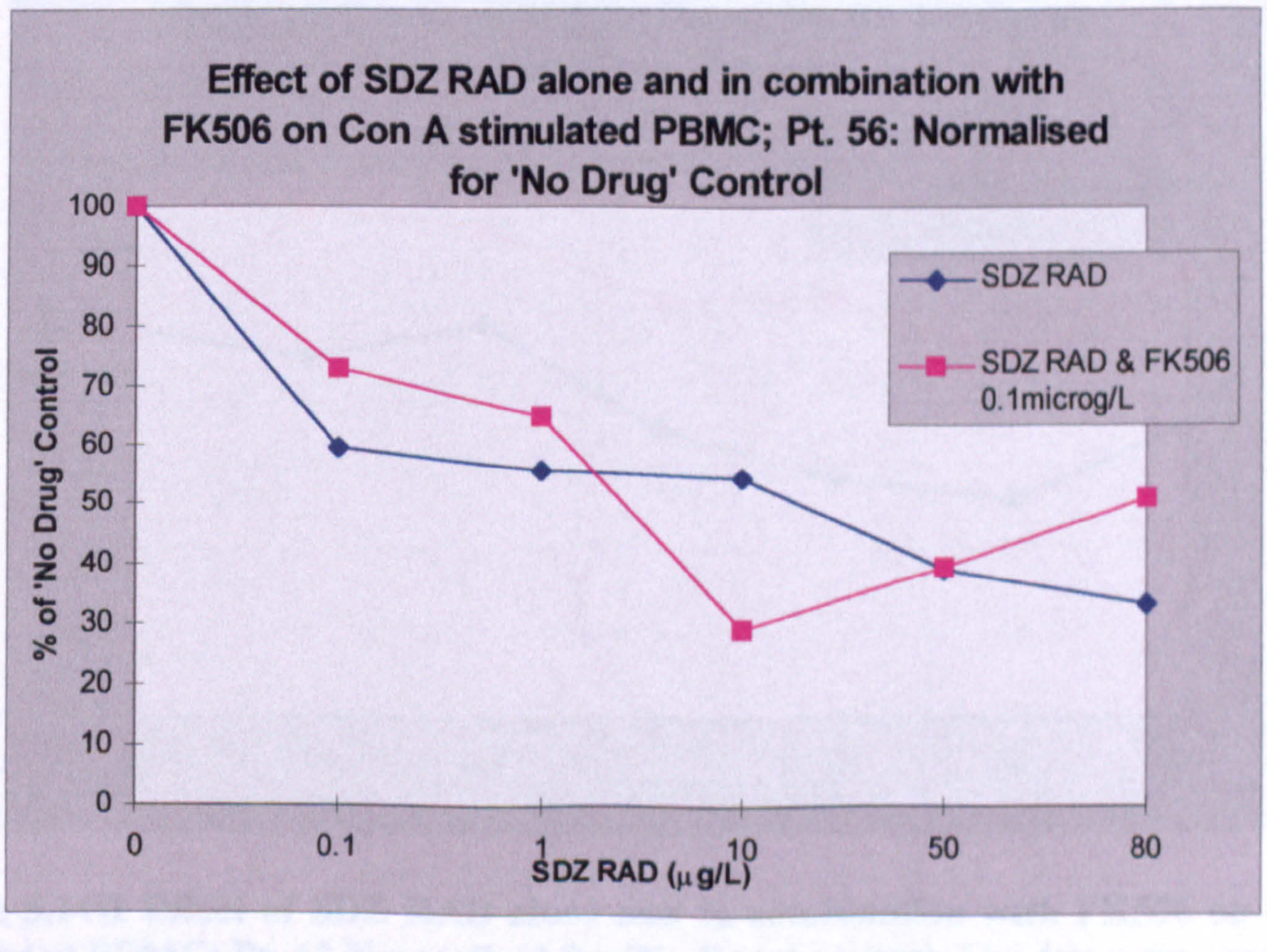


Figure 5.14F Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC; Pt. 56: Normalised for 'No Drug' control. The data were normalised by assigning '100%' to the level of proliferation obtained in the absence of any drug and calculating the percentage of inhibition achieved with respect to the 100% level of proliferation for both SDZ RAD alone and SDZ RAD in combination with FK506.

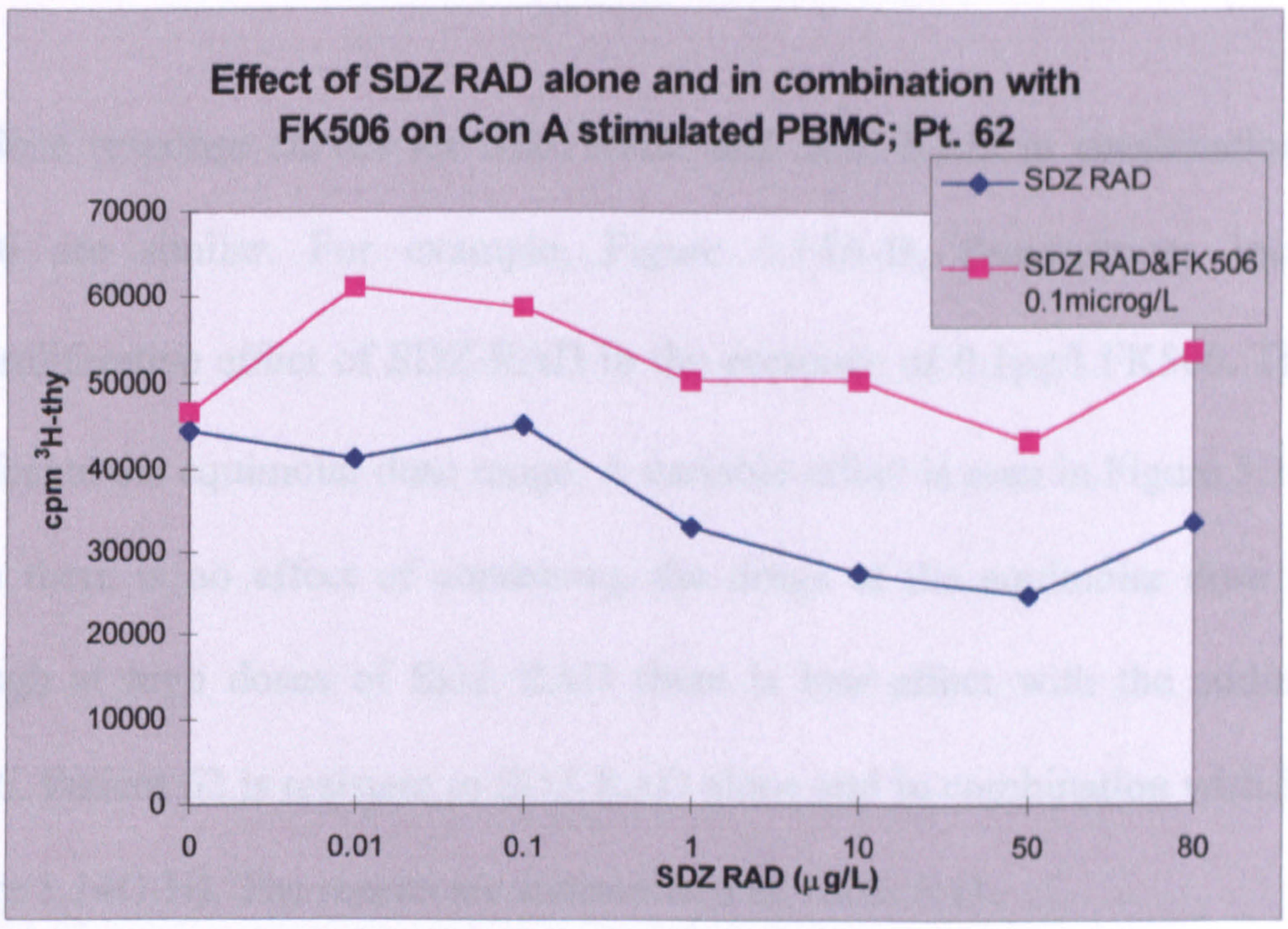


Figure 5.14G Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC. PBMC from a single patient were incubated in the presence or absence of SDZ RAD and FK506, as indicated, for 48 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations.

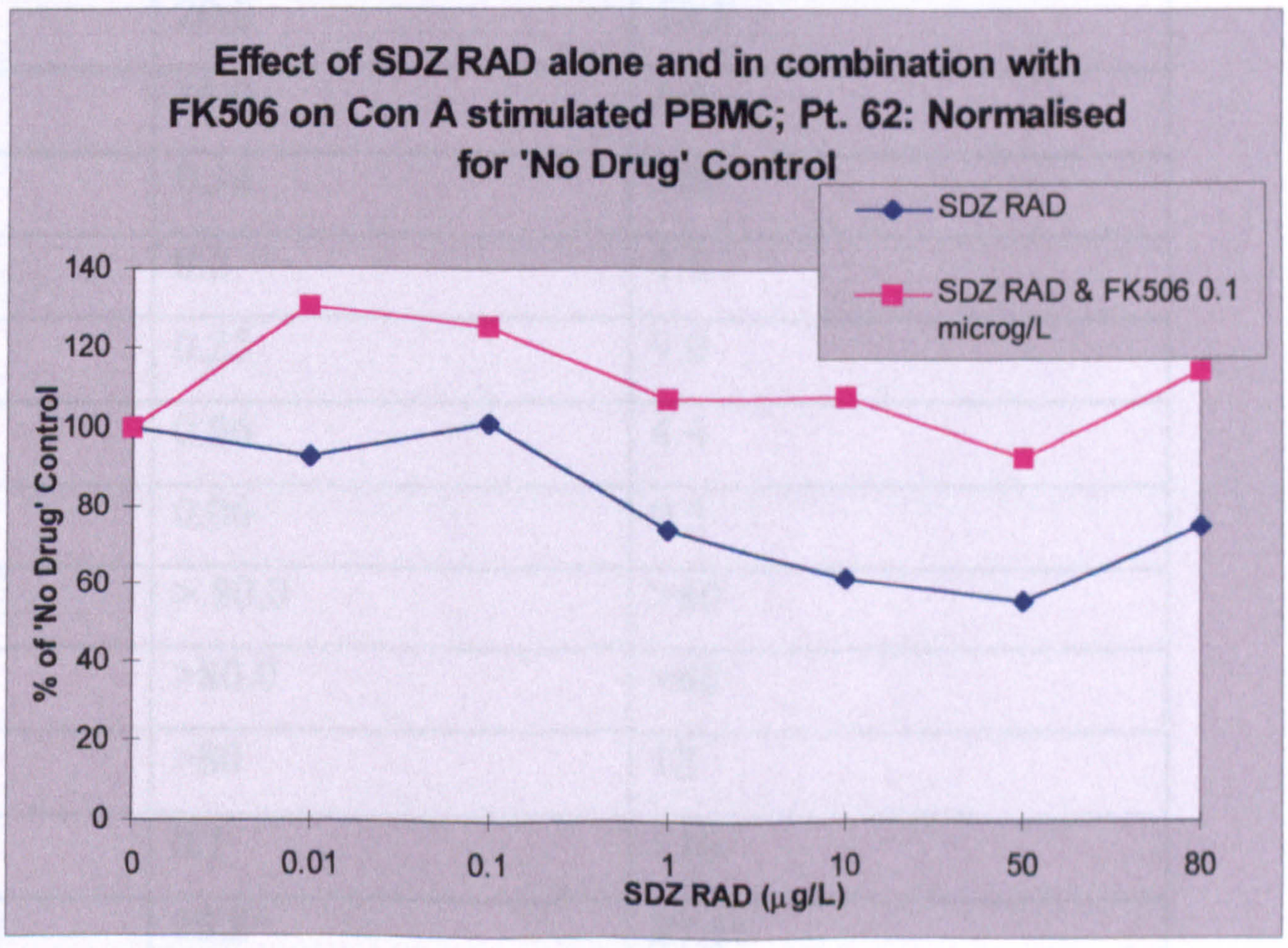


Figure 5.14H Effect of SDZ RAD alone and in combination with FK506 on Con A stimulated PBMC; Pt. 62 Normalised for 'No Drug' control. The data were normalised by assigning '100%' to the level of proliferation obtained in the absence of any drug and calculating the percentage of inhibition achieved with respect to the 100% level of proliferation for both SDZ RAD alone and SDZ RAD in combination with FK506.

The dose response curves for SDZ RAD and SDZ RAD in combination with FK506 are similar. For example, Figure 5.14A-B, demonstrates increased anti-proliferative effect of SDZ RAD in the presence of 0.1µg/l FK506. There is no effect at the equimolar dose range. A variable effect is seen in Figure 5.14C-D. Again there is no effect of combining the drugs at the equimolar dose range, although at high doses of SDZ RAD there is less effect with the addition of FK506. Patient 62 is resistant to SDZ RAD alone and in combination with FK506 (Figure 5.14G-H). The results are summarised in Table 5.11.

Patient No.	**ID_{50} SDZ RAD (µg/l)**	**ID_{50} SDZ RAD & FK506 (µg/l)**
53	20.0	10.5
56	16.0	4.5
57	0.34	·80
58	0.2	1.5
63	0.25	9.0
59	0.06	4.4
9	0.06	0.3
64	> 80.0	·80
62	>80.0	·80
50	>80	18
41	0.1	·10
Mean	**25.2***	**27.1***
SD	**35.9**	**34.3**

Table 5.11 ID_{50}s for SDZ RAD alone and SDZ RAD in combination with FK506. ID_{50}s were established Con A stimulated PBMC cultures for SDZ RAD and SDZ RAD in combination with FK506.

Footnote to Table 5.11. Paired 2 tail t Test comparing each group p 0.85
If '>80' then '80' entered into equations; if '·10' then '10' entered into equation

The mean ID_{50} for SDZ RAD alone is 25.2µg/l (SD 35.9 range .06-80). The mean ID_{50} for SDZ RAD used in combination with FK506 is 27.1µg/l (SD 34.3 range 0.3-80). There is no statistical difference between the two groups. However, there appears to be 2 anomalous results that may be skewing the results (patients 50 and 57). If these results are withheld from the analysis the respective mean ID_{50}s are 21.9µg/l (SD 33.8) and 22.2µg/l (SD 32.9). (p 0.87). Despite the lack of effect across the group this hides some marked differences between the patients. In 3 patients the addition of FK506 rendered the cells more sensitive to the effects of SDZ RAD. In 2 patients there was no overall demonstrable effect, although these patients were resistant to the effects of SDZ RAD. In the remaining 6 patients the cells became less sensitive to the effects of SDZ RAD following the addition of FK506.

Conclusion

There were no consistent effects of combining SDZ RAD with FK506 in Con A stimulated cells.

5.3.2 MLR

Introduction

The MLR was performed by co-culturing recipient PBMC with PBMC from surrogate donors matched at the DR locus as discussed in Sections 3.1.3 and 4.2.5. The MLR offers the opportunity to study the response of PBMC from renal transplant patients with a physiological stimulus. The level of proliferation obtained is low in comparison to the broad stimulus provided by mitogen.

During these experiments one of the two populations of PBMC were rendered incapable of proliferation following pre-treatment with mitomycin C. These treated cells are termed 'stimulators' (S). The untreated cells remain capable of proliferation and serve as 'responders' (R). The responder cells were renal transplant recipients and the stimulator cells were matched for the original kidney donor at the DR locus. The recipient characteristics including HLA typing are included in Table 4.2.

5.3.2.1 Preliminary experiment to investigate duration of culture and optimum cell number in 1-way MLR

A 1-way MLR was established with varying duration of cultures (108-156 hours) and comparing the response of 1 x 10^5cells/culture to 0.5 x 10^5 cells/culture. The MLR containing 1 x 10^5 cells from both donor and recipient did very poorly and

did not survive (data not shown). When 0.5×10^5 cells from both (S) and (R) were established in the MLR there was a time dependent effect with no response at the longest duration. The best response was seen at 108 hours (Figure 5.15).

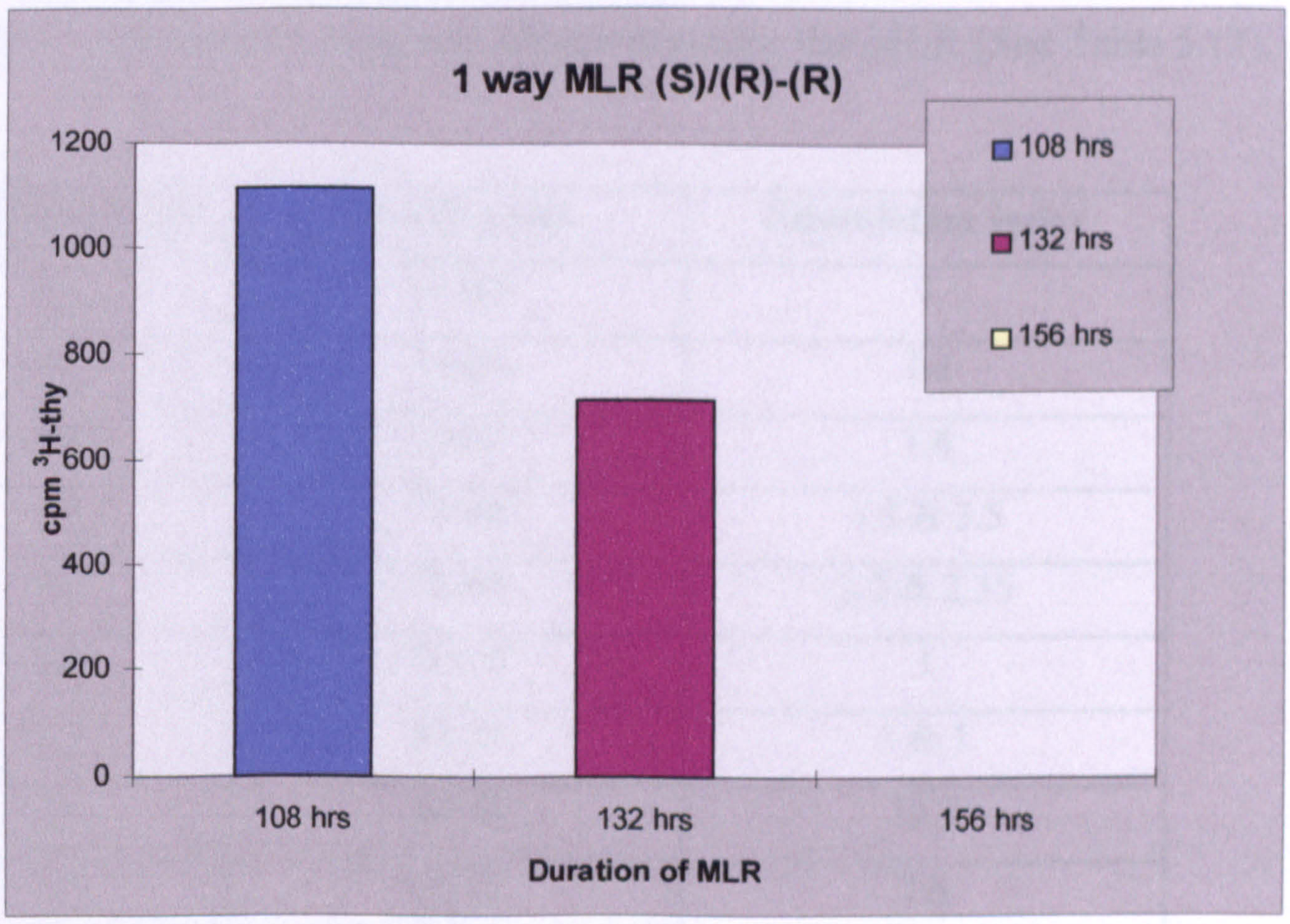


Figure 5.15 Bar Chart of ^{3}H-thymidime incorporation against duration of MLR Pt.No.68 1-way MLR were established in cell cultures for 108, 132 and 156 hours with the addition of ^{3}H-thymidine 16 hours before the end of the culture. After harvesting, ^{3}H-thymidime incorporation was determined and the data are presented as means of replicate determinations.
Footnote: (S)/(R) refers to stimulator and responder combination in MLR. The responder only (R) results have been deducted.

5.3.2.2. Quantifying the proliferative response

The surrogate donor/recipient combinations tested were characterised as to the degree of proliferative response obtained during the MLR (See Table 5.12).

Experiment No.	(S)/(R) pairs	Stimulation Index
A1	46:65	1
A2	78:66	1.4
A3	39:67	1.6
A4	79:68	3.5 & 3.5
A5	80:69	2.2 & 2.35
A6	80:70	1
A7	82:54	1 & 1
A8	80:46	10.5
A9	81:55	2.6
A10	82:54	4.1
A11	84:69	7.1
A12	70:21	6.2
A13	65:28	3.6
A14	55:60	6.8
A15	83:69	3.7 & 18.7
A16	85:54	17.5
A17	64:59	19.5
A18	85:61	14.6

Table 5.12 Summary of the stimulation index (SI) for each stimulator/responder (S)/(R) pair. The SI was calculated from each pair by dividing the value obtained with the stimulator/responder pair by that of the responder cells alone. i.e. (S)(R)/(R).

The (S)/(R) pairs from A1-A9 show relatively low stimulation, except for A8. On 3 occasions in this series the MLR were repeated and consistent results were obtained.

Following the use of the supplemented medium A10-A18 the levels of stimulation increased to maximum stimulation index of 19.5. It is possible that the medium initially used was not fully sustaining for these MLR experiments. It is interesting to note, however, that despite this the cells responded well to Con A when added after 72 hours of culture.

5.3.2.3 Investigation of effect of FK506 on MLR

FK506 was added in graded doses from 0.01-5μg/l to the MLR and the cultures were maintained as described previously (see Section 4.2.5). The data for patient 65 are presented in Table 5.13

	(S) Only	(R) Only	Con A	(S)/(R) FK506 (μg/l)					
				0	**0.01**	**0.1**	**0.3**	**1**	**5**
	62	1565	8969	1553	48	28	6	0	22
	48	1588	9335	982	633	90	50	36	168
				819	455	16	16	28	114
Mean cpm ^{3}H-thy		**1577**	**9152**	**1118**	**378.7**	**44.7**	**24**		**101.3**
SD				**385.4**	**299.9**	**39.7**			**73.8**

Table 5.13 Effect of FK506 on MLR: pt. 65 1-way MLR was established in the presence of graded doses of FK506 (0.01-5μg/l). Proliferation was assessed by incorporation of ^{3}H-thymidine. [(S) refers to stimulator cells; (R) refers to responder cells from patient 65]

The mitomycin treated cells (S) do not proliferate at all, and this is a consistent finding throughout the experiments. The response to Con A stimulated controls is maintained, and this is consistent throughout the experiments.

Data from a further patient (number 66) is presented graphically as in Figure 5.16.

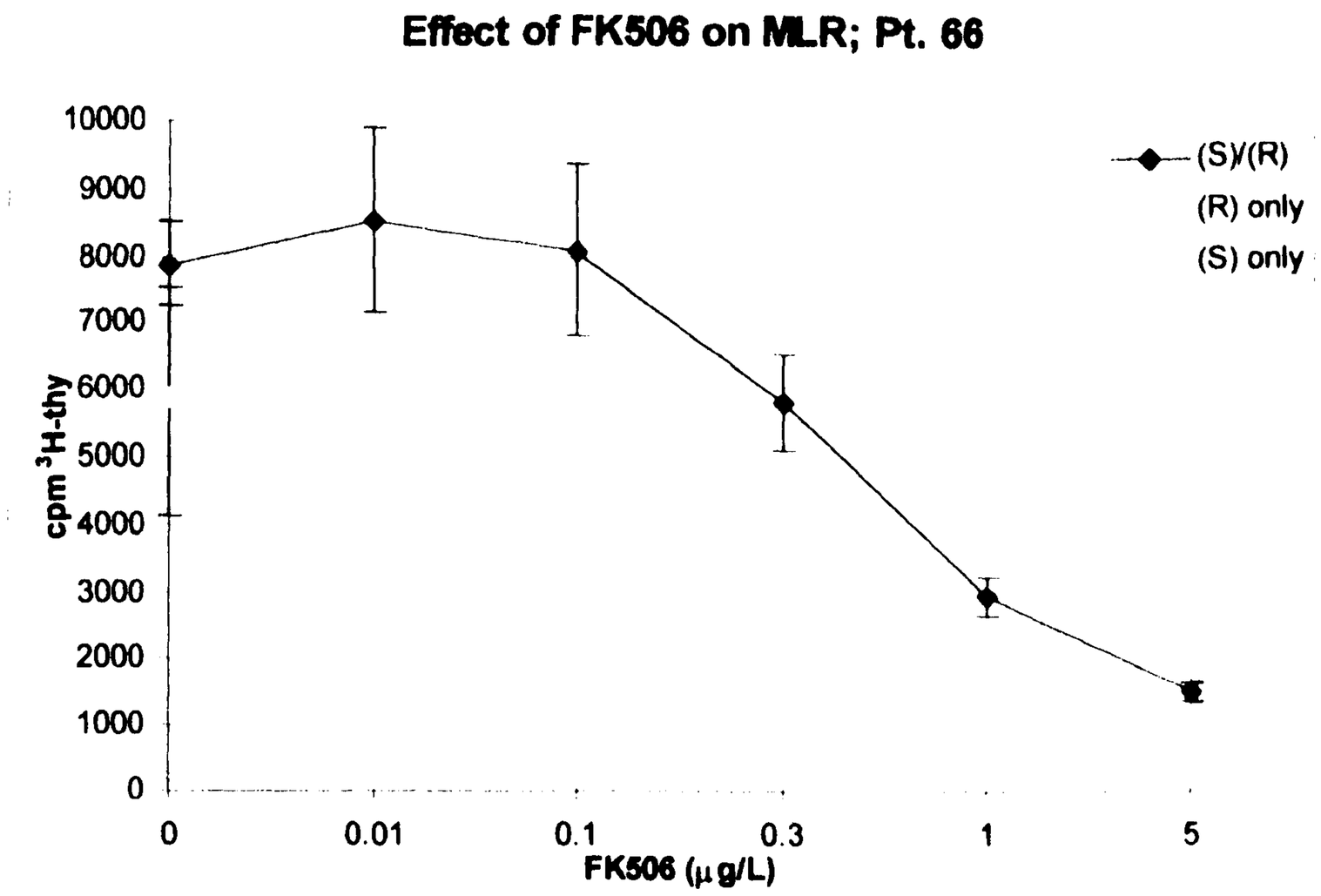


Figure 5.16 Effect of FK506 on proliferative response during the MLR. 1-way MLR were established in the presence or absence of FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars represent the standard deviation around a mean of 3 values.

A dose response curve is obtained during the MLR in response to FK506. However, there is a significant amount of background stimulation seen in the Responder (R) only control cells. The stimulator (S) cells however, do not proliferate since they have been pre-treated with the anti-mitotic agent mitomycin C.

5.3.2.4 Investigation of the effect of SDZ RAD on MLR

1-way MLR were established in the presence of graded doses of SDZ RAD (0.01-5µg/l) as discussed in Section 4.2.5. Data from 3 patients is presented graphically in Figure 5.17A-C.

Data from a further patient (number 66) is presented graphically as in Figure 5.16.

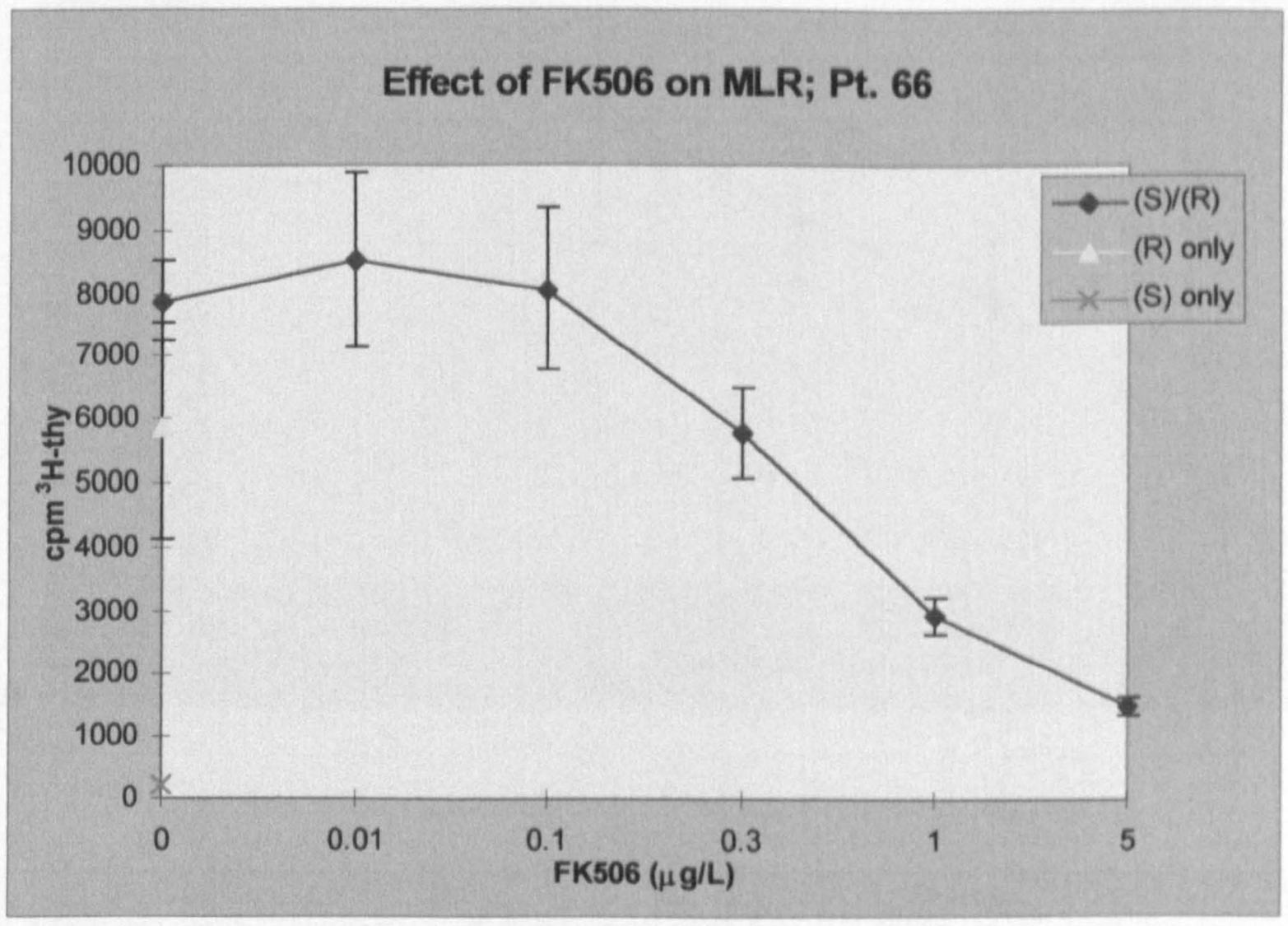


Figure 5.16 Effect of FK506 on proliferative response during the MLR. 1-way MLR were established in the presence or absence of FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars represent the standard deviation around a mean of 3 values.

A dose response curve is obtained during the MLR in response to FK506. However, there is a significant amount of background stimulation seen in the Responder (R) only control cells. The stimulator (S) cells however, do not proliferate since they have been pre-treated with the anti-mitotic agent mitomycin C.

5.3.2.4 Investigation of the effect of SDZ RAD on MLR

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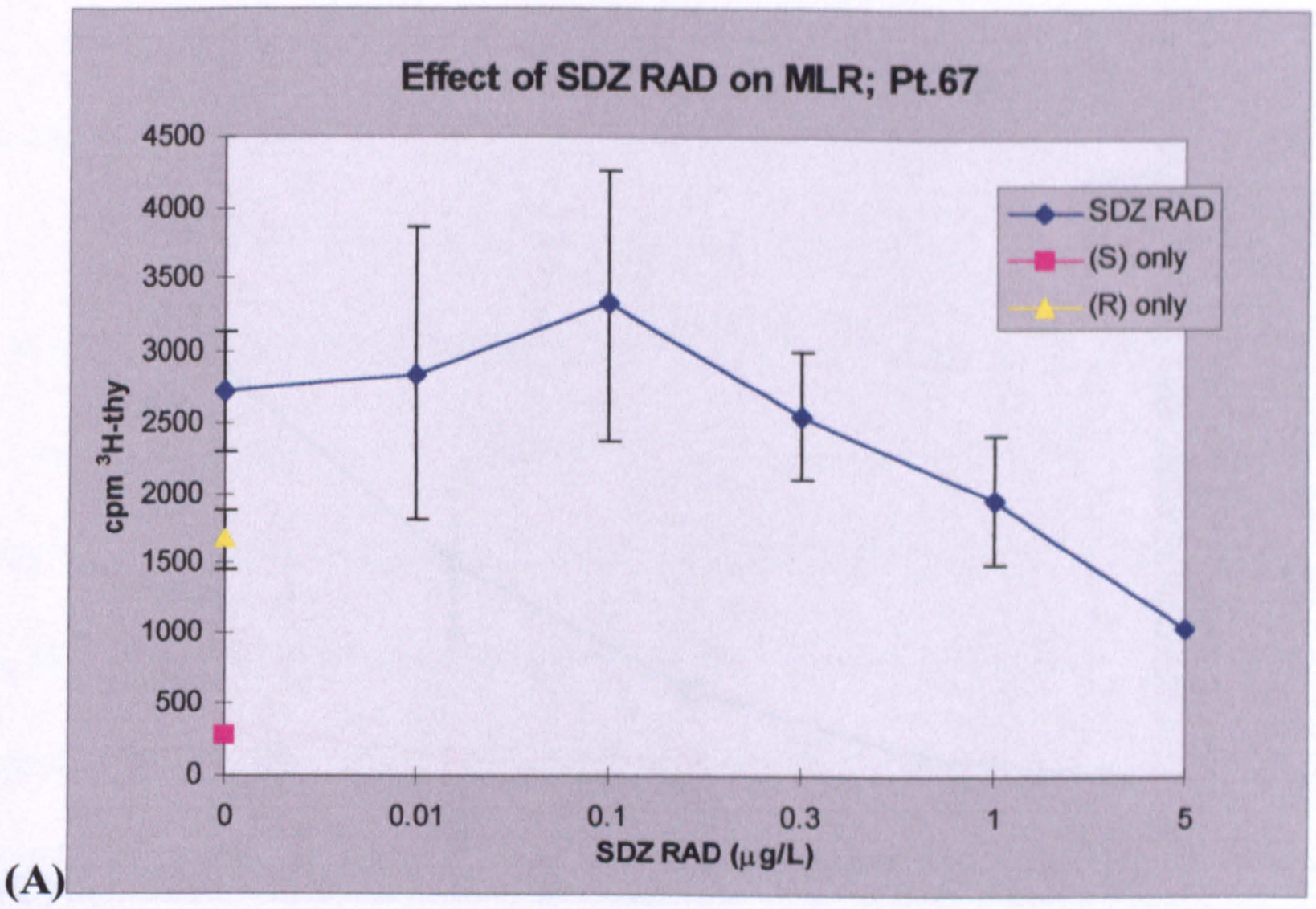
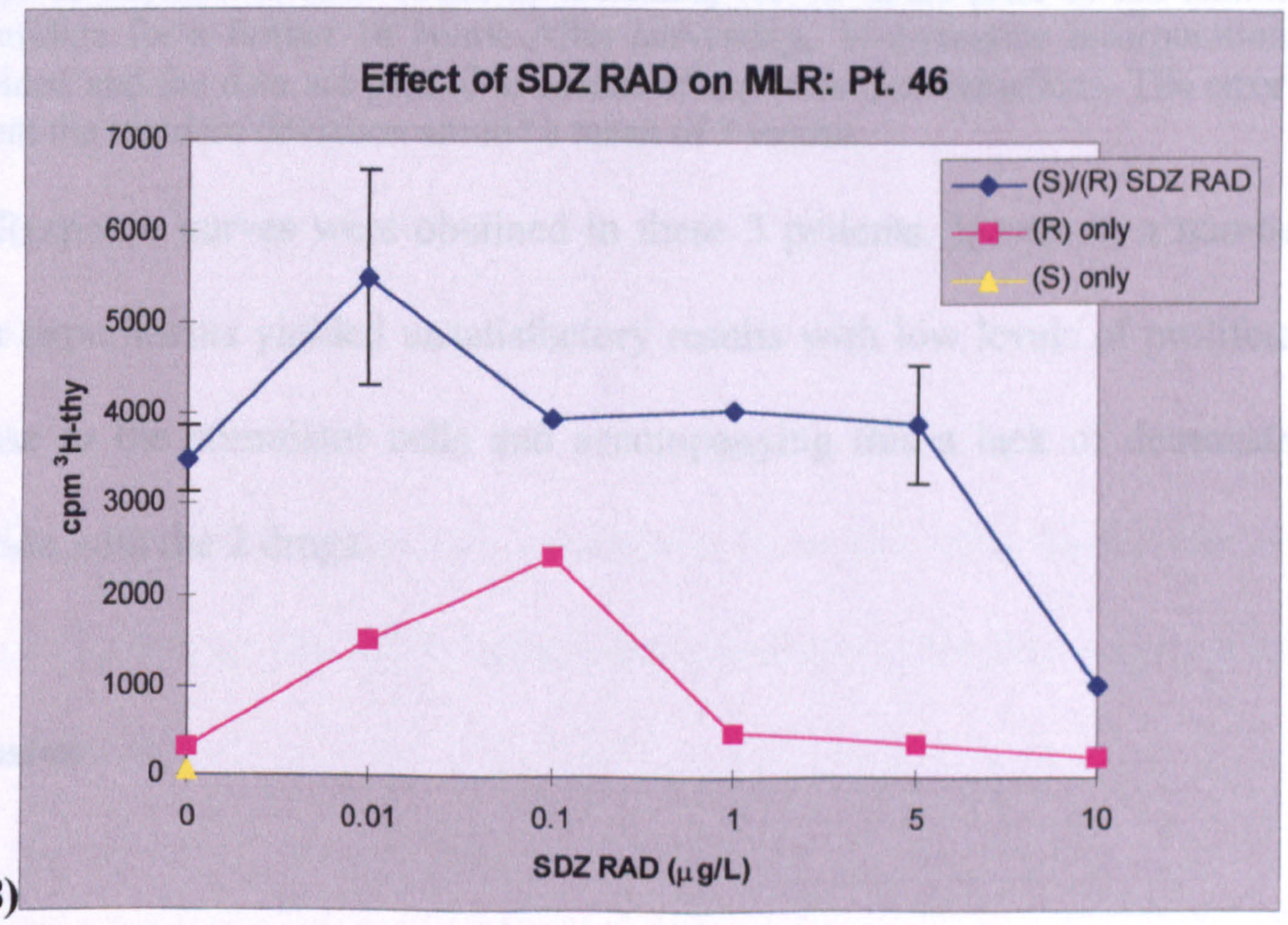

Effect of SDZ RAD on MLR; Pt.67
cpm ³H-thy
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
0.01
0.1
0.3
1
5
SDZ RAD (μg/L)
SDZ RAD
(S) only
(R) only

(A)


Effect of SDZ RAD on MLR: Pt. 46
cpm ³H-thy
7000
6000
5000
4000
3000
2000
1000
0
0
0.01
0.1
1
5
10
SDZ RAD (μg/L)
(S)/(R) SDZ RAD
(R) only
(S) only

(B)

(C)

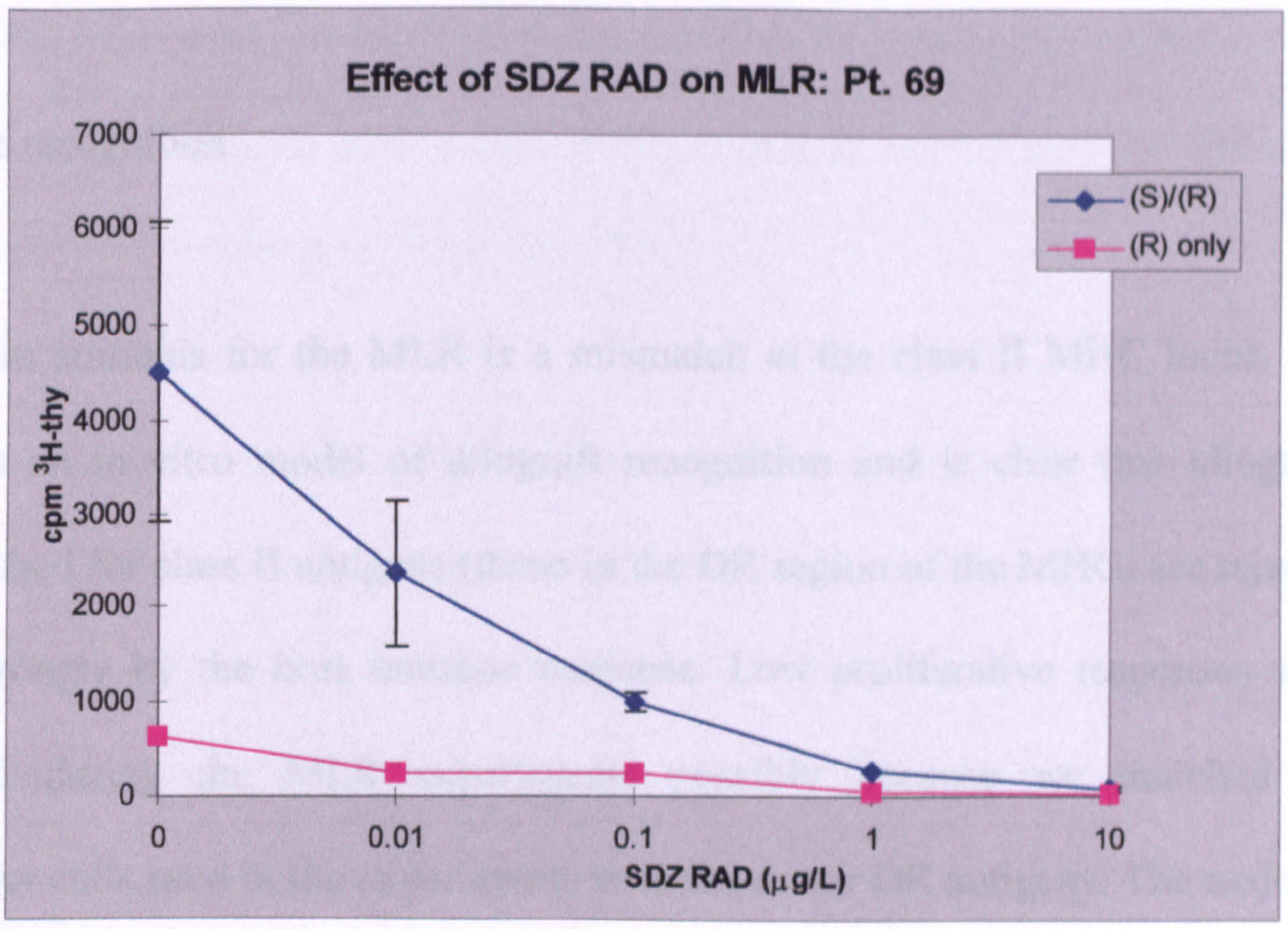


Figure 5.17A-C Effect of SDZ RAD on MLR. 1-way MLR were established in the presence or absence of SDZ RAD, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars represent the standard deviation around a mean of 3 values.

Dose-Response curves were obtained in these 3 patients. However, a number of further experiments yielded unsatisfactory results with low levels of proliferative response to the stimulator cells and accompanying this a lack of demonstrable inhibition with the 2 drugs.

Discussion

There was evidence of a poor proliferative response in a number of MLR. The poor responses obtained during the MLR made it more difficult to interpret the inhibitory effects of the drugs and may have accounted for the lack of pharmacological effect of combining the two drugs in *in vitro* lymphocyte cultures. There are a number of possible explanations for the observed poor responses.

Immune recognition

The main stimulus for the MLR is a mismatch at the class II MHC locus. The MLR is an in vitro model of allograft recognition and is clear that allografts mismatched for class II antigens (those in the DR region of the MHC) are rejected most strongly by the host immune response. Low proliferative responses were obtained during the MLR experiments possibly because we matched the stimulator cells used in the experiments with the donor DR antigens. The majority of transplant recipients in the experiments are matched at this locus (See Table 4.2). The MLR performed when there is matching for Class II MHC alleles do not react or proliferate briskly.

Technical

(a) Despite repeated washes the mitomycin C treated cells may have led to contamination of the MLR, which abrogated a proliferative response in the responder cells.

(b) The medium used for the experiments may not have been sustaining the cell cultures. The medium used for the early experiments did not contain HEPES buffer or β-mercaptoethanol (β-ME) that serves as an anti-oxidant. The later experiments were characterised by improved proliferative responses and the medium at this stage contained both HEPES and β-ME. However, at all stages when the cells were tested for proliferative response to Con A at day 3 of

culture, there was always a brisk proliferative response, suggesting that the media used during the experiments were sustaining, even during the prolonged culture duration associated with the MLR.

(c) The cells, although in general were able to respond to Con A following freezing and thawing, may have lost the ability to respond to a more specific stimulus. Again there is the anomaly of cells responding poorly to the specific allogeneic stimulus during the MLR, but continuing to respond well when Con A is introduced into the cultures after 3 days.

5.3.2.5 Effect of SDZ RAD and FK506 used in combination on MLR

The effect of combining the two drugs on the dose response curves obtained during the MLR was assessed (section 4.2.3). The MLR were established with SDZ RAD added to the cultures in graded doses, either alone, or with a fixed amount of FK506 (between 0.01-1.0 μg/l). Data from these experiments are presented graphically in Figure 5.18. It can be seen from Figure 5.18A that the curves are similar. However, they start from different values of ^{3}H-thymidine incorporation. The graphs are normalised by nominating the amount of proliferation seen with neither drug present ('No Drug' Control) as 100%. Any change in proliferation above or below this level is compared to this level. Both curves now begin from the same point of 100%. Figure 5.18B is a graph of the same patient but with normalised data points.

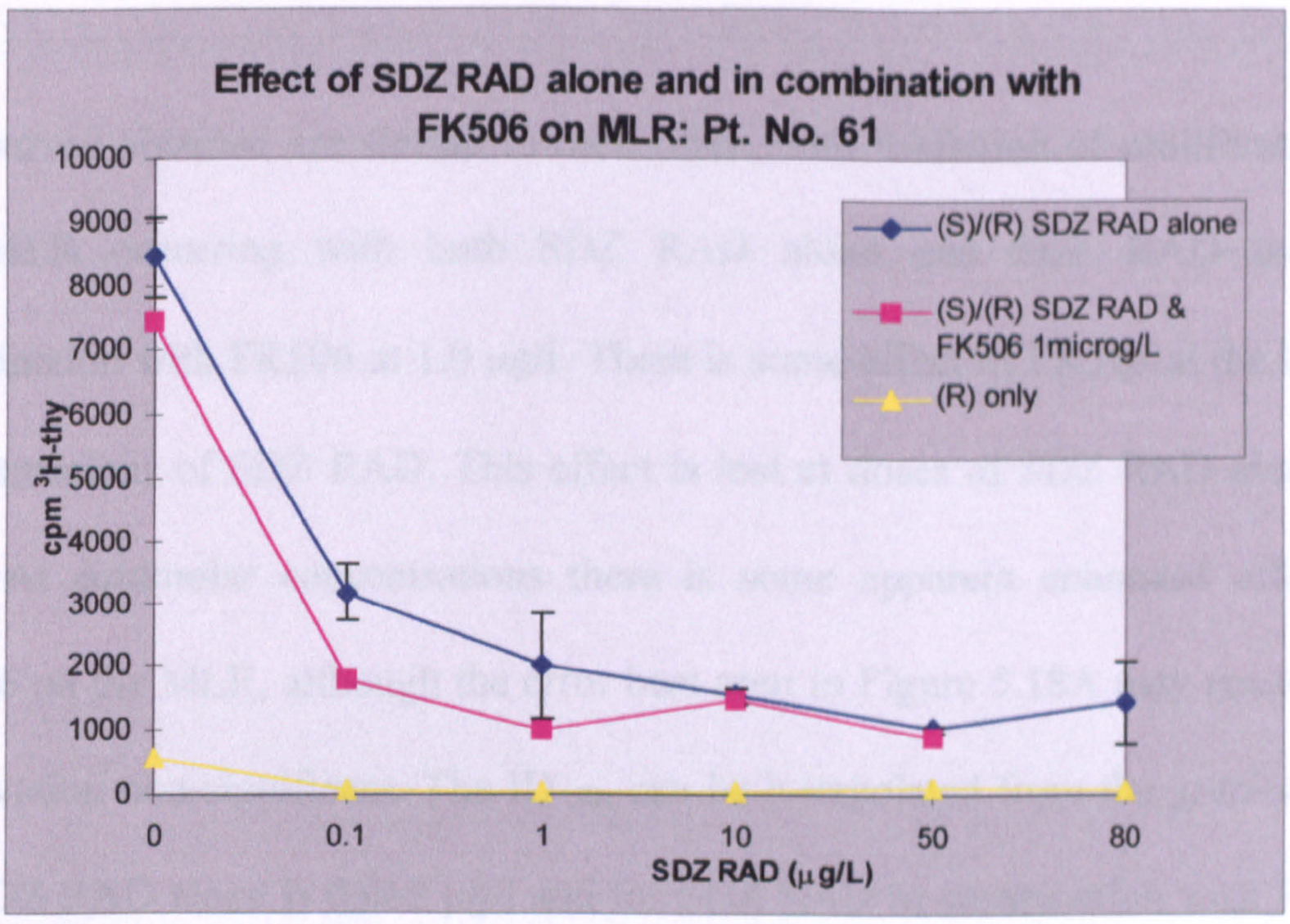


Figure 5.18A Effect of SDZ RAD alone and in combination with FK506. 1-way MLR were established in the presence or absence of SDZ RAD and FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined and the data are plotted as means of replicate determinations. The error bars represent the standard deviation around a mean of 3 values. Responder (R) only cells are treated in the same way.

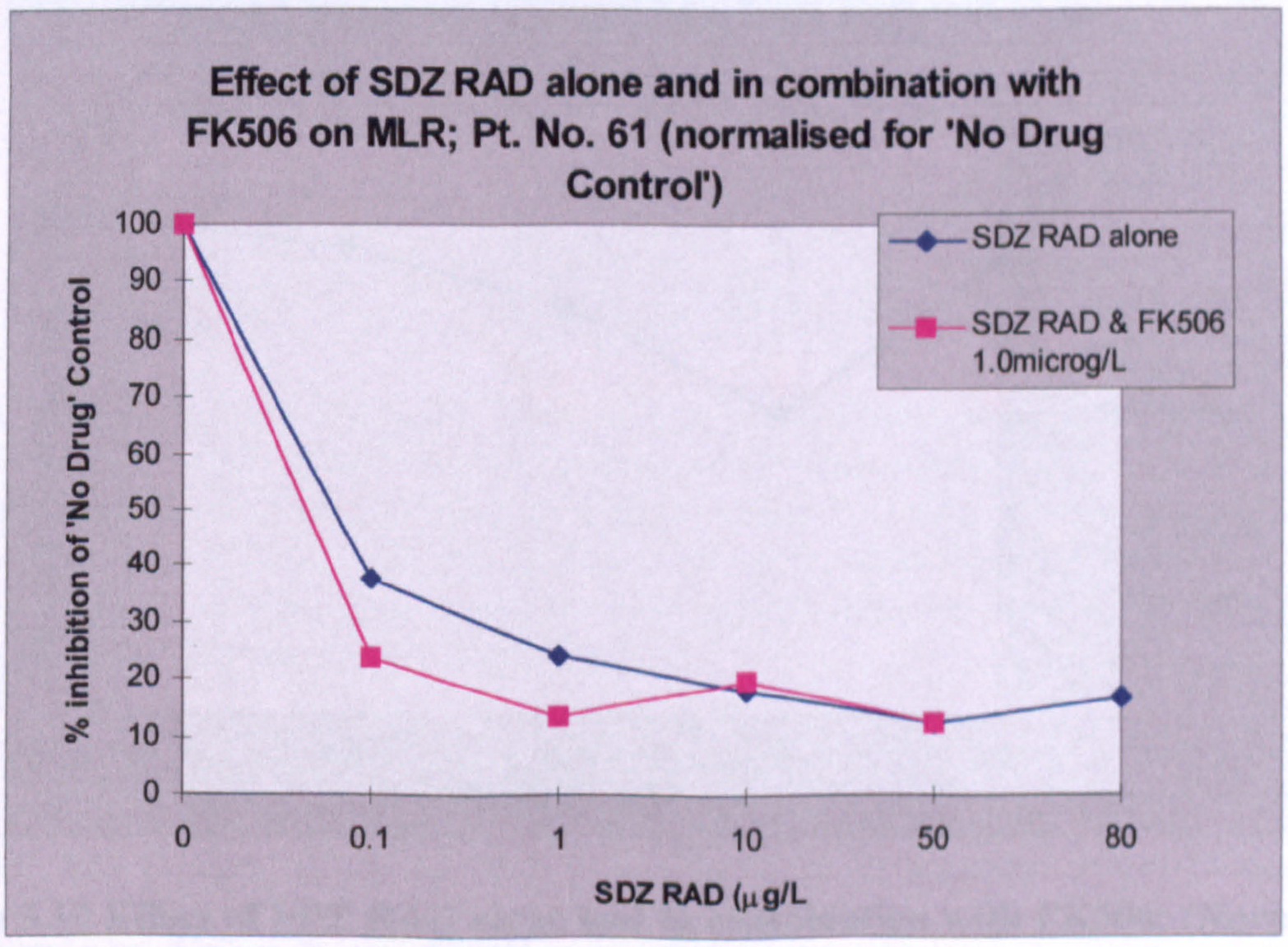


Figure 5.18B Normalisation curve of effect of SDZ RAD alone and in combination with FK506 on MLR: (Normalised for 'No Drug' control). 1-way MLR were established in the presence or absence of SDZ RAD and FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined. The data are normalised such that the 2 curves commence from '100%'. This is the level of proliferation obtained when neither SDZ RAD nor FK506 are present in the cell cultures. ID $_{50s}$ can be estimated by interpolation of the graphs.

The curves obtained are similar to each other, with inhibition of proliferation of the MLR occurring with both SDZ RAD alone and SDZ RAD used in combination with FK506 at 1.0 μg/l. There is some effect of FK506 at the lowest concentrations of SDZ RAD. This effect is lost at doses of SDZ RAD above 10 μg/l. At equimolar concentrations there is some apparent enhanced effect of FK506 on the MLR, although the error bars seen in Figure 5.18A may render this observation non-significant. The ID_{50s} can be interpolated from the graphs (ID_{50} for SDZ RAD alone is 0.062 μg/l and for SDZ RAD in combination with FK506 is 0.042 μg/l). Normalised data from 2 further patients are presented in Figures 5.19 and 20.

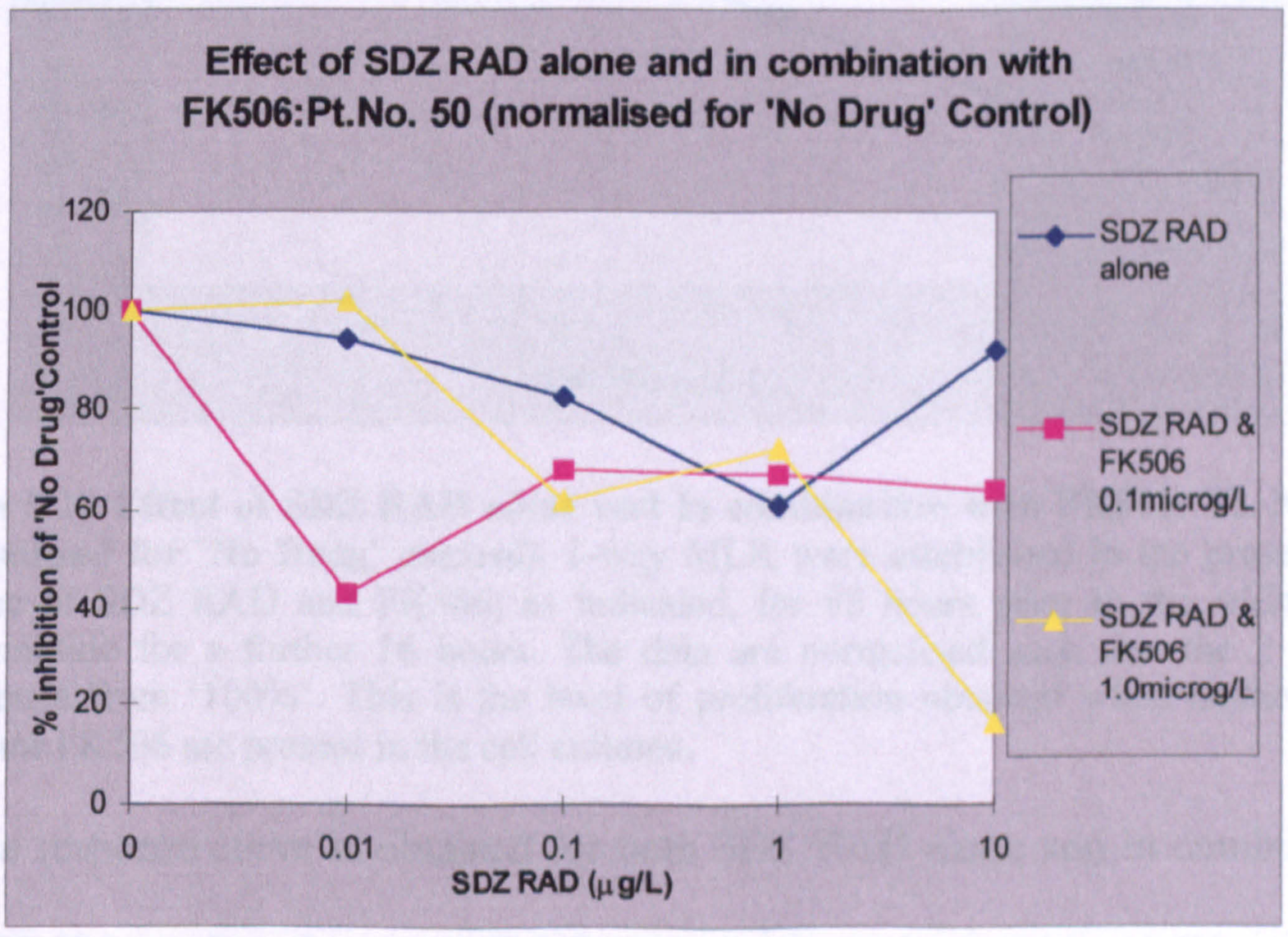


Figure 5.19 Effect of SDZ RAD alone and in combination with FK506: (Normalised for 'No Drug' control). 1-way MLR were established in the presence or absence of SDZ RAD and FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. After harvesting, ^{3}H-thymidine incorporation was determined. The data are normalised such that the 2 curves commence from '100%'. This is the level of proliferation obtained when neither SDZ RAD nor FK506 are present in the cell cultures. ID_{50s} can be estimated by interpolation of the graphs.

At equimolar doses (0.1 and 1.0 μg/l) the curves for SDZ RAD alone and SDZ RAD in combination with FK506 come together. The greatest effect on inhibition (greater than 80%) occurs at the highest doses of each drug used in combination (SDZ RAD 10 μg/l and FK506 1.0 μg/l). When SDZ RAD is used alone the curve does not reach the 50% of inhibition level and therefore an ID_{50} cannot be estimated. Data from patient 86 is presented in Figure 5.20.

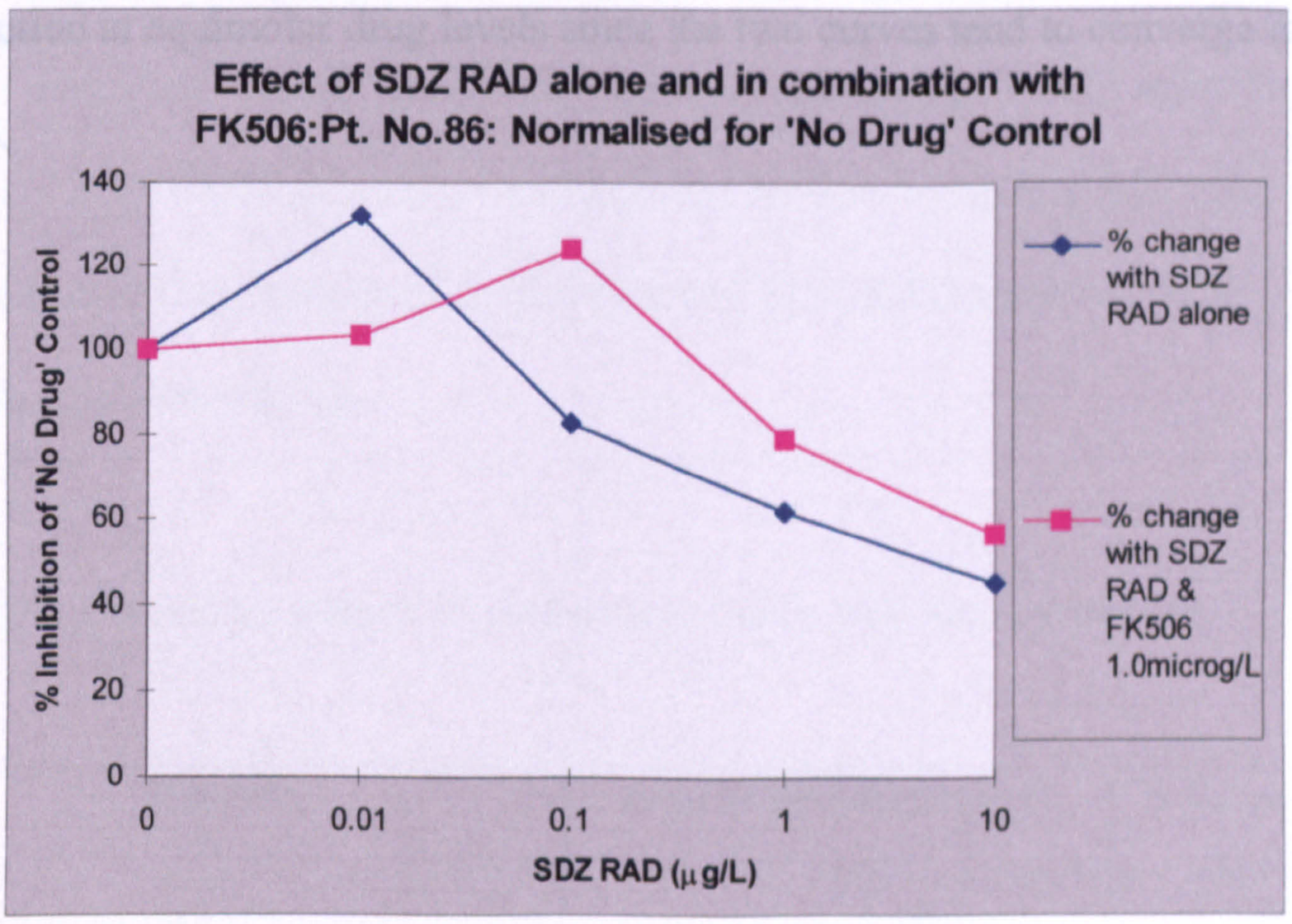


Figure 5.20 Effect of SDZ RAD alone and in combination with FK506: Pt. No. 86: (Normalised for 'No Drug' control). 1-way MLR were established in the presence or absence of SDZ RAD and FK506, as indicated, for 92 hours prior to the addition of ^{3}H-thymidine for a further 16 hours. The data are normalised such that the 2 curves commence from '100%'. This is the level of proliferation obtained when neither SDZ RAD nor FK506 are present in the cell cultures.

A dose response curve is obtained for both SDZ RAD alone and in combination with FK506. At low doses of SDZ RAD there is an increase in proliferation of cells as measured by the percentage inhibition being greater than 100%. The effect of the drug combination appears to be less than that when SDZ RAD is used alone. The overall level of inhibition is sub maximal and although a dose response is seen from 0.01μg/l with SDZ RAD alone, and 0.1μg/l when used in

combination with FK506, doses of SDZ RAD greater than 10 µg/l would have been necessary for maximal inhibitory effect.

Summary

There are no consistent effects of combining SDZ RAD with FK506 on the MLR from renal transplant recipients. Of particular note there is no evidence of interaction at equimolar drug levels since the two curves tend to converge at these levels.

5.3.3 CD25 expression

Cell proliferation can be assessed by following incorporation of 3H-thymidine as demonstrated in the data presented in the preceding sections. The immunosuppressant drugs studied inhibit T cell activation in response to alloantigens. CD25 [α-chain of the Interleukin-2 Receptor (IL-2R)] is a marker of T cell activation that is specifically upregulated during T cell activation (see Section 3.1.5). CD25 expression was assessed by flow cytometry following Con A stimulation in the presence of graded doses of SDZ RAD alone and SDZ RAD in combination with FK506.

The data obtained for patient 55 is shown in Table 5.14 and Figure 5.21.

Test cells	Dose of SDZ RAD (μg/l)	% Of cells staining for CD25
Unstimulated Cells/Isotype control	0	0.2
Stimulated Cells/Isotype control	0	0.5
Unstimulated Cells/CD25 stained	0	0.3
Stimulated Cells/CD25 stained	0	90
Stimulated Cells/ CD25 stained	0.01	87.2
Stimulated Cells/ CD25 stained	0.1	68.5
Stimulated Cells/ CD25 stained	1.0	30
Stimulated Cells/ CD25 stained	10.0	32.4
Stimulated Cells/ CD25 stained	50.0	38.3

Table 5.14 Effect of SDZ RAD on expression of CD25 following Con A stimulation of PBMC from a renal transplant patient. PBMC were stimulated with 5μg/ml Con A for 60 hours in the presence of graded doses of SDZ RAD (0.01-50μg/l). The cells were harvested and stained with anti-CD25 monoclonal antibody. An isotype control antibody was used in the experiment. The cells were examined for CD25 expression by flow cytometry and the percentage of positive cells was obtained.

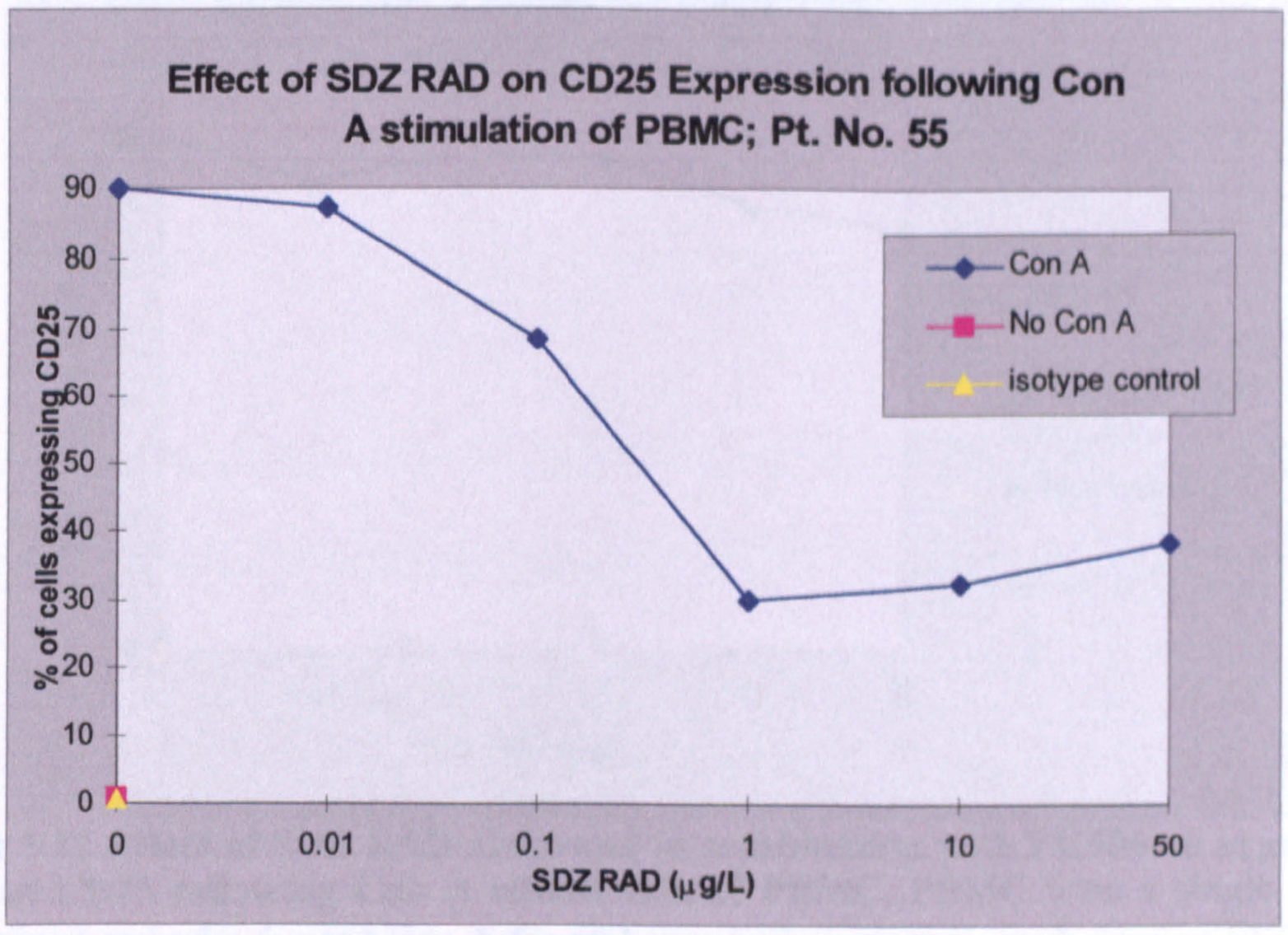


Figure 5.21 Effect of SDZ RAD on expression levels of CD25 following Con A stimulation of PBMC from a renal transplant patient. PBMC from a single patient were stimulated with 5µg/ml Con A for 60 hours in the presence or absence of SDZ RAD as indicated. The cells were harvested and stained with anti-CD25 monoclonal antibody and isotype control antibody. The cells were examined for CD25 expression by flow cytometry and the percentage of positive cells obtained.

The percentage of cells expressing CD25 is progressively reduced following the addition of SDZ RAD up to a dose of 1µg/l. The level of CD25 expression in non-stimulated cells is almost zero. CD25 expression on stimulated PBMC in the presence of SDZ RAD and FK506 was also examined in 2 further patients (Figures 22 and 23). There is no evidence of inhibition of CD25 in patient 58 following the addition of SDZ RAD alone or in combination with FK506 (Figure 5.22). It is therefore not possible to assess any interaction between the two drugs. Patient 57 is similarly resistant to SDZ RAD and FK506 as assessed by CD25 expression. The expression of CD25 is not inhibited by SDZ RAD alone or in combination with FK506 except when FK506 is added at high concentration (Figure 23).

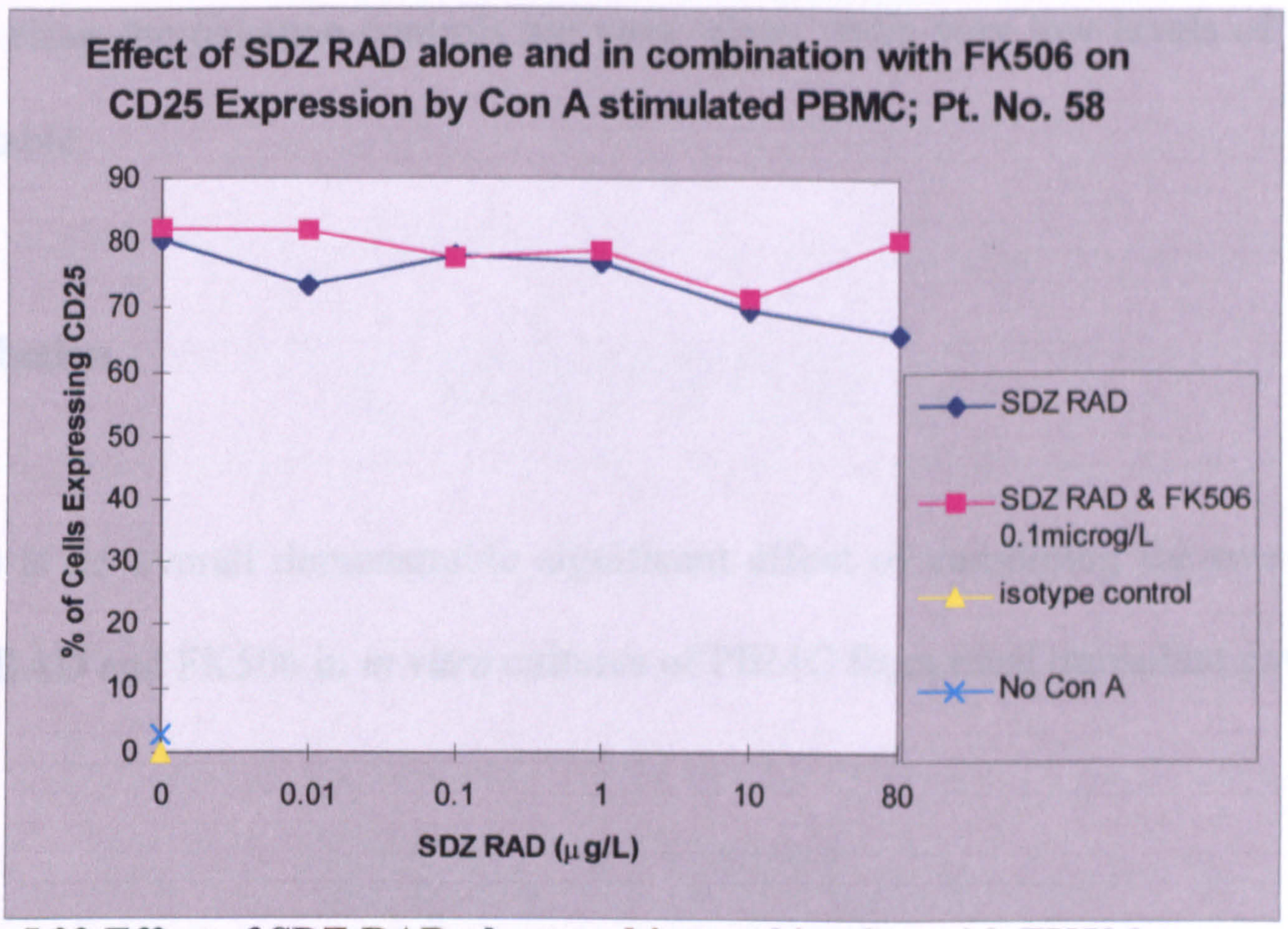


Figure 5.22 Effect of SDZ RAD alone and in combination with FK506 on expression levels of CD25 following Con A stimulation of PBMC. PBMC from a single patient were stimulated with 5µg/ml Con A for 60 hours in the presence or absence of SDZ RAD as indicated. The cells were harvested and stained with anti-CD25 monoclonal antibody and isotype control antibody. The cells were examined for CD25 expression by flow cytometry and the percentage of positive cells obtained.

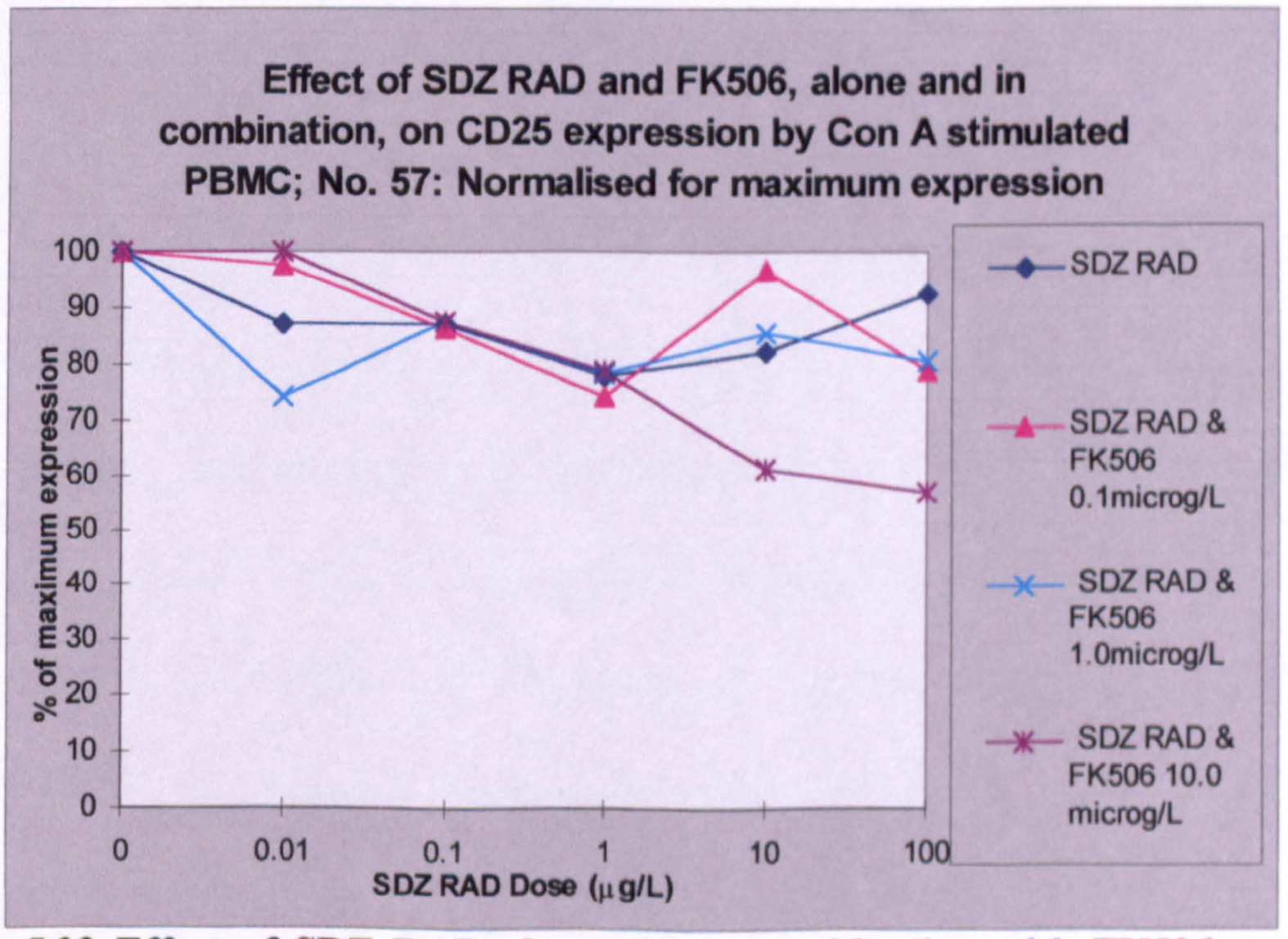


Figure 5.23 Effect of SDZ RAD alone and in combination with FK506 on CD25 expression by Con A stimulated PBMC. (Normalised for maximum expression). PBMC from a single patient were stimulated with 5µg/ml Con A for 60 hours in the presence or absence of SDZ RAD as indicated. The cells were harvested and stained with anti-CD25 monoclonal antibody and isotype control antibody. The cells were examined for CD25 expression by flow cytometry and the percentage of positive cells obtained. The data are normalised for the maximum expression obtained with no drug present in the culture.

In all cases the negative controls are very 'clean' with very low levels of CD25 detectable.

Conclusion

There is no overall demonstrable significant effect of combining the two drugs SDZ RAD and FK506 in *in vitro* cultures of PBMC from renal transplant patients.

CHAPTER 6

DISCUSSION

6.1 Lymphocyte sensitivity to Cy A and FK506

To investigate the range of variation of lymphocyte sensitivity to Cy A and FK506 assays were performed using mitogen stimulation *in vitro* mononuclear cell cultures from patients who had received a renal transplant. The data confirm a wide variation in the response of peripheral blood mononuclear cells (PBMC) to Cy A and FK506 both quantitatively and qualitatively section 5.1). There was a correlation between Cy A and FK506 sensitivities between patients. This implies that in general if the cells are sensitive to Cy A then they will be sensitive to FK506. The data, however, also concealed some considerable variation between patients in terms of the ratio of sensitivities to either drug. This was found to be dependent on the drug used in the patient's treatment protocol and is suggestive of the development of selective resistance to the drug received by the individual patient. The two independent associations described suggest that more than one mechanism may determine drug sensitivity at the cellular level. The inter-patient correlation of ID_{50}s for the two drugs implies that there may be common mechanisms to account for drug sensitivities since both drugs act via calcineurin inhibition. However, the development of selective resistance to either drug suggests other mechanisms may be important. The stated hypothesis explored during this thesis that the intracellular drug concentration is modulated by multidrug resistance mechanisms will be discussed in section 6.2.

The initial impetus for this study was the observation that patients rejecting their kidney transplants with apparently therapeutic levels of Cy A could have graft function salvaged by conversion from Cy A to another Calcineurin inhibitor,

namely, FK506 or Tacrolimus (Jordan *et al.*, 1994 and 1997). The explanation for this effect is not clear but would appear to be multifactorial. Factors likely to be important in explaining this phenomenon have been explored during this thesis. To begin a discussion of the putative factors explaining this observation it is necessary to describe the basic pharmacological principles relating to the use of the drugs Cy a and FK506.

The pharmacological effects of a given drug are defined by the pharmaceutical, pharmacokinetic and pharmacodynamic properties of the drug. Briefly, the pharmaceutical properties of a drug are those associated with the formulation of the drug for administration and the route of administration. The pharmacokinetic properties of a drug relate to absorption of the drug following administration, whether given orally or by another route (for example transdermal, buccal, or by inhalation etc) and the subsequent metabolism, distribution and elimination from the body. After oral administration the amount of drug that reaches the systemic circulation depends on its absorption and the extent to which it escapes metabolism in the gastrointestinal tract and liver and this is its systemic availability or bioavailability. The pharmacodynamic properties of the drug are the effects that the drug has on individual cells that lead to the pharmacological effects of the drug. The pharmacodynamic effects of a drug are determined by the ability of the drug to interfere with target 'receptors' within the cell or on the cell membrane. The drug-receptor interaction can be stimulatory or inhibitory. All of these factors are relevant when considering the observation of Cy A 'failure' in allograft rejection. Firstly, as discussed in section 1.4.2 pharmacokinetic profiling of Cy A has evolved from the introduction of Cy A in the 1980s to the present

day. During the studies reported by Jordan *et al* in 1994 and 1997 the formulation of Cy A administered to patients was conventional Cy A or Sandimmun®. This formulation is an incomplete emulsification of Cy A that produces wide variations in bioavailability. Since Cy A has a narrow therapeutic window it is important to perform therapeutic drug level monitoring in blood samples taken from patients. The widely practiced method of monitoring has been trough or pre-dose level monitoring (C-0). However, it has been demonstrated that trough levels do not predict clinical outcomes in terms of acute rejection episodes and one-year graft survival (Kahan *et al.*, 1996). This observation may give a pharmacokinetic explanation for the apparent Cy A failure seen with conventional Cy A in renal transplant recipients despite apparently adequate therapeutic drug levels. The subsequent formulation, Neoral® was introduced which is a microemulsion with improved absorption characteristics compared to conventional Cy A. Data from Canadian Investigators has recently shown that trough blood levels of Cy A when the Neoral® formulation is administered do not predict those patients in whom acute rejection occurs although high trough levels are associated with increased nephrotoxicity (Mahalati *et al.*, 1999). Conversely, the absorption profile of FK506 demonstrates a wide inter-individual variability but with a close association of trough blood levels with clinical events (Kershner and Fitzsimmons, 1996). These factors relating to the pharmacokinetics of the drugs offer one explanation for the improved efficacy of FK506 over conventional Cy A seen in the 'salvaging' trials reported by Jordan.

Both Cy A and FK506 are potent inhibitors of T cell activation in response to an immunological stimulus via the inhibition of the phosphatase enzyme, calcineurin.

Both drugs mediate their effects on calcineurin following binding to their respective intracellular receptors, cyclophilin and FK506 binding protein (FKBP). Both of these molecules, known collectively as immunophilins, are present at high levels within cells. The T cell response to these drugs is central to their pharmacological effects and this led to the hypothesis that variation in response of T cells from renal transplant recipients to Cy A and FK506 may exist. It was not clear whether the individual responses to the two drugs would be the same or independent. Extrapolating these questions to the 'salvaging data' the hypothesis that individual patients cells were either resistant, or had developed resistance, to the effects of Cy A. The data presented in this thesis data support this as a potential mechanism.

In vitro inhibition of mitogen induced lymphocyte proliferation by both Cy A and FK506 has been reported on cells from healthy volunteers and uraemic dialysis patients (Hibbins *et al.*, 1993). These studies confirmed that FK506 is effective in concentrations approximately 30-100 times lower that of Cy A and that there is wide inter-individual variability. The data presented in this thesis is an extension of the above, having been performed on renal transplant patients' cells, and confirms the wide variability in sensitivity. Measurement of lymphocyte sensitivity may define drug failure (i.e. rejection occurring despite therapeutic blood levels) more scientifically. The current widespread clinical use of FK506 for "Cy A failure" has no scientific validity. Patients with trough Cy A blood levels of about 200 µg/l are converted to FK506 at starting levels of about 15 µg/l, which means that if FK506 is about 30 times more potent that Cy A, the same immunosuppressive result might have been achieved by increasing the Cy A level

to 450 µg/l. The advantage of FK506 could therefore solely be due to its relative dose, and perhaps a better therapeutic index (ratio of desired effect to adverse effects).

Comparison of the ID_{50}s and serum concentrations suggests that although in most cases there is a reasonable excess, some patients may be underdosed i.e. trough blood concentration may be close to the ID_{50} and others may be overdosed i.e. the blood concentration may be many times the ID_{50}. The question arises as to whether this is of clinical significance. Underdosing implies potentially inadequate immunosuppression: whilst overdosing would exacerbate side effects. One difficulty in assessing the data is that the trough blood levels do not represent the peak drug level or area under the curve (AUC). Comparing ID_{50} for either drug between patients who did or did not suffer acute rejection, no difference was found. However, the number of patients rejecting was very low (seven). Likewise, there was no correlation between sensitivities and blood creatinine levels, nature of therapy (triple therapy with prednisolone against therapy without prednisolone) or time since transplant. Since the study was not set up to examine these particular aspects then the sample size of patients may have been too small to see a correlation with clinical parameters.

The observation of selective resistance developing to the drug used for treatment is novel and interesting. Longitudinal studies measuring sensitivities of PBMC from renal transplant patients may elucidate the time after a transplant that resistance develops. This would be important clinically since if resistance could be predicted the patients could be switched to alternative treatment. It is possible that

a new approach to anti-rejection protocols could be established based on the observation of selective resistance that would allow tailoring of treatment for individual patients. Should resistance develop to each drug used for treatment then protocols that utilise rotating immunosuppressant would be necessary.

6.2 Do multidrug resistance (MDR) mechanisms account for the variation in sensitivity to Cy A and FK506?

The stated hypothesis that the development of selective drug resistance occurs through the intracellular drug concentration being modulated by multidrug resistance mechanisms has not been proven, although there are a number of reasons for it to be an attractive model. As discussed in section 1.6.6, both Cy A and FK506 are substrates for at least one of the mechanisms implicated in multidrug resistance to cancer chemotherapy. This is the P-glycoprotein encoded by the MDR1 locus (Saeki *et al.*, 1992), a membrane protein that pumps hydrophobic substances out of cells (Ueda *et al.*, 1987). This is expressed by subsets of lymphocytes (Klimecki *et al.*, 1994) and Con A-stimulated mouse spleen cells have been shown to become more sensitive to doxorubicin in the presence of P-gp inhibitors (Pourtier-Manzanedo *et al.*, 1992).

Despite a report in the literature (Pilarski *et al.*, 1995) that P-gp is readily detected on the surface of normal lymphocytes none of the patients tested in this study had even moderate levels of P-gp detectable on the surface of their lymphocytes with the monoclonal antibodies 4E3 and MRK16. These antibodies were chosen since they recognise external epitopes on the cell surface and can be applied to unfixed

cells in suspension. The lack of MRK16 staining in this study may reflect insufficient sensitivity to detect P-gp rather than non-expression. Another group found little expression of P-gp on peripheral lymphocytes despite attempting to increase sensitivity by using biotinylated MRK16 (Takeshita *et al.*, 1995). Similarly it has been shown that P-gp mRNA can be detected despite the absence of immunological expression of P-gp (Ludescher *et al.*, 1992). A factor that may influence antibody binding in this experimental system includes distortion of the external epitope. The possibility that unfixed cells may have distorted the target epitope or internalised the epitope was investigated. Experiments were performed on cells fixed with formaldehyde/acetone. By fixing the cells it was hypothesised that the epitope would be fixed on the external surface of the cell and would not be subject to post antibody binding modification. However, the results of these experiments confirmed that antibody binding to the cells could not be demonstrated. Whether the process of cryoprecipitation and thawing of cells was disrupting the epitope sites was explored by repeating the experiments using fresh cells. There was no evidence of P-gp expression during these experiments. Further experiments with higher concentrations of the primary monoclonal antibody and different secondary conjugates similarly yielded no significant binding to PBMC. Clearly, the positive control cell line MCF7 exhibited high levels of P-gp expression confirming the antibodies were working. However, this breast cancer cell line has been selected for high levels of P-gp expression by culture in medium containing adriamycin and may not bear any resemblance to PBMC from renal transplant patients.

Despite the lack of detectable P-gp expression, all the patients tested in this study exhibited P-gp functional activity as demonstrated by R-123 dye efflux. R-123 is a fluorescent dye that diffuses into the cell and if not actively transported by an efflux pump is trapped in the cell and accumulates within mitochondria. Approximately 42% of total normal adult PBMC export the dye and this decreases with age (Pilarski *et al.*, 1995). The dye efflux is secondary to P-gp activity since specific inhibitors such as the cyclosporin analogue, PSC 833, readily inhibit it. All patients exhibited similar levels of cells actively pumping and there was no difference in pumping activity in those patients where the Con A stimulated lymphocytes were sensitive to Cy A and FK506 compared to those where the cells were resistant to the drugs. This indicates that there is no gross difference between the drug sensitive and drug insensitive PBMC in P-gp function. However, the majority of the experiments were performed on bulk cell populations rather than subsets of cells. In those experiments where subsets of cells were analysed it was demonstrated that CD8 positive cells more actively pumped than CD4 positive cells and this is consistent with an earlier report (Pilarski *et al.*, 1995). One could speculate that the responsive cells in the PBMC cultures stimulated with Con A are primarily CD4 positive and therefore it was unlikely that a vast difference in pumping activity between high and low sensitivity groups would be observed since the background level of pumping is relatively low.

The observation of P-gp functional activity in PBMC from renal transplant patients raises the possibility of P-gp activity affecting the proliferation of cells in response to Cy A and FK506. In these experiments with PBMC from renal transplant patients there was no demonstrable effect on sensitivity to Cy A or

FK506 following the addition of selective P-gp inhibitor, PSC 833. The patients had received treatment with either drug as part of their immunosuppressive regime and it may be that MDR1 gene expression and P-gp function is down-regulated following prolonged treatment with the drugs, perhaps in a similar fashion that P-gp expression can be induced with rifampicin (Greiner *et al.*, 1999). Indeed, it has been shown previously that no patients who had transplants for more than 40 months expressed the MDR1 gene in their lymphocytes (Gotzl *et al.*, 1995).

Characterisation of the MDR1 gene has intensified recently with the findings of functional polymorphisms and the correlation of one allele with P-glycoprotein expression and activity (Hoffmeyer *et al.*, 2000). Analysis of the MDR1 sequence in 21 volunteers was performed. P-gp expression and functional activity in intestinal mucosa had also been determined. There was a significant correlation of a polymorphism in exon 26 (C3435T) of the MDR1 gene with expression levels and function of the MDR1 phenotype. The homozygous 'T'-allele is associated with more than two fold lower MDR1 expression levels compared with homozygous CC samples. This finding was consistent when the function of P-gp was assessed.

Could variation in the frequency of this polymorphism have an effect on P-gp expression and function in cells from lymphoid lineages? A report has suggested that the C3434T mutation in the MDR1 gene may alter the functional characteristics of P-gp in CD56+ natural killer cells, as assessed by rhodamine 123 efflux (Hitzl *et al.*, 2001). Although, clearly, the patients in this present study have not been examined for MDR1 polymorphisms it would be an interesting area

FK506 following the addition of selective P-gp inhibitor, PSC 833. The patients had received treatment with either drug as part of their immunosuppressive regime and it may be that MDR1 gene expression and P-gp function is down-regulated following prolonged treatment with the drugs, perhaps in a similar fashion that P-gp expression can be induced with rifampicin (Greiner *et al.*, 1999). Indeed, it has been shown previously that no patients who had transplants for more than 40 months expressed the MDR1 gene in their lymphocytes (Gotzl *et al.*, 1995).

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for future study. A population-based survey has been performed to analyse the frequency of the C3435T polymorphism of the MDR1 gene in different ethnic groups (Schaeffeler *et al.*, 2001). The results reveal that 83% of West Africans compared to 26% of Caucasians are homozygous for the C allele. It is proposed by these investigators that the consequences of higher intestinal P-gp expression due to the increased frequency of the CC genotype in Africans could be that bioavailability of drug substrates of P-gp could be reduced. This has been confirmed for Cy A (Min *et al.*, 2000). The population studied in this thesis was predominantly of Caucasian origin and this may explain the low levels of expression and function of P-gp observed.

The calcineurin inhibitors are highly lipophilic and therefore can readily enter cells. Since the intracellular receptors for the drugs are abundant within a cell, for example, FKBP proteins constituting between 0.3-0.5% of cellular proteins (Siekierka *et al.*, 1989) then complexes of drug/immunophilin will be formed readily. In addition to this, experiments utilising an FK506-derived radioactive ligand confirm that half maximal T cell suppression occurs following occupancy of FKBP12 of only 3-5% of the available pool (Dumont *et al.*, 1994). For P-gp to modulate the intracellular concentration of Cy A and FK506 to such an extent as to affect their anti-proliferative effects in these circumstances would require the molecule to be very highly expressed and functionally active. Experiments to test the hypothesis that increased expression of P-gp confers protection against Cy A dependent inhibition of calcineurin (CN) phosphatase activity have been performed on selected cell lines expressing varying levels of P-gp (Fakata *et al.*, 1998). The study group utilised cells of the human epidermal carcinoma cell line

KB-V that were grown in media supplemented with varying strengths of vinblastine. Dose response curves were then obtained for CN phosphatase activity against Cy A concentration. KB-V cells grown in high dose vinblastine shifted the dose response curve to the right some 70-fold compared to the parent KB cells. Verapamil, a P-gp inhibitor, completely extinguished the protection afforded by P-gp expression. The cell line reported by this group was highly selected in vinblastine and although this offers a model of how P-gp may affect sensitivity to Cy A, the cells used are not directly comparable to the patients' cells examined in this thesis. Alternative mechanisms to explain variations in lymphocyte sensitivity need to be considered. Both drugs act by binding to intracellular receptors of the immunophilin type (Bierer, 1994). The differences in drug response could be accounted for in differences in the amounts of these proteins in cells from different donors. Downregulation of cyclophilin levels in T lymphocytes from Cy A-treated patients, and likewise of FKBP in FK506-treated patients, is one possible explanation for the selective drug resistance observed. There is some evidence that both cyclophilins and FKBP proteins are regulated genes, for example varying in their expression with differentiation status (Chatellard-Gruaz *et al.*, 1994 and Lyons *et al.*, 1995), but as yet no evidence that this may influence the response of cells to Cy A or FK506. Due to time and resource constraints there was no opportunity to undertake this area of study.

Non P-gp phenotypes of MDR, namely, MRP and LRP have been described. The detection of MRP mRNA in normal haemopoietic cells from peripheral blood and bone marrow has been demonstrated as well as MRP protein expression determined with specific monoclonal antibodies (Legrand *et al.*, 1996). Functional

assays of MRP have been explored (Den Boer *et al.*, 1997). The clinical data regarding MRP is sparse and difficult to interpret since the methods used to characterise the expression and functional activity are not standardised. The data pertaining to LRP suggest that LRP is not detectable on normal lymphocytes (Michieli *et al.*, 1997). This data led to the conclusion that the most relevant system to study was P-gp dependent mechanisms of MDR. Nevertheless, further investigation of MRP in normal lymphocytes and lymphocytes from renal transplant recipients may be warranted. In summary P-gp dependent mechanisms do not appear to explain variations in lymphocyte sensitivities to the immunosuppressant drugs Cy A and FK506 and the data indicate that several mechanisms may be of importance in this area.

As outlined in the Preface to this thesis, the idea behind this work was to develop a means of studying *in vitro* patient lymphocyte responses to immunosuppressive drugs in order to understand their mode of action, mechanisms of resistance, and interactions between them. Such an understanding may allow for future prescribing of rational, patient-specific immunosuppressive drug protocols. Having described their mode of action, and the investigation of mechanisms of resistance, the data relating to the interactions between the macrolide FK506 and the rapamycin analogue SDZ RAD is considered. FK506 and SDZ RAD share the same intracellular binding sites but have different anti-proliferative targets within the cell. The early studies on rapamycin confirmed mutual antagonism with FK506 and led to the theory of a shared intracellular binding site (Dumont *et al.*, 1990a). However, a study in a rat cardiac transplantation model did not demonstrate antagonism but an enhanced immunosuppressant effect of combining

rapamycin with FK506 (Vu *et al.*, 1997). This polarisation of effect raised the question of how would the drugs interact in *in vitro* cultures of lymphocytes from renal transplant patients. This is a worthwhile area of study since the potential of using two drugs with predictable pharmacokinetic profiles in a synergistic way may lead to safer and more efficacious anti rejection protocols.

6.3 Interaction of FK506 and SDZ RAD in *in vitro* lymphocyte cultures from renal transplant patients.

Lymphocyte cultures were established in both Con A driven systems and by Mixed Lymphocyte Reaction (MLR). Lymphocyte proliferation in response to Con A was inhibited in a dose dependent manner following the addition of either FK506 or SDZ RAD in the majority of cases. Considering the data with SDZ RAD alone there was a wide range of sensitivity with values of ID_{50} from 0.009-80 μg/l. However, subgroup analysis of 9 patients confirms a mean ID_{50} of 4.7μg/l with a range of values from 0.009-20μg/l. This figure for SDZ RAD is approximately three fold higher than that obtained with FK506 in the previous experiments, and is also in the same order of magnitude as that reported with *in vitro* data comparing SDZ RAD and rapamycin (Schuler *et al.*, 1997). The reasons for the wide variation in sensitivities to SDZ RAD are unclear. It is possible that certain cell samples from individual patients are inherently less sensitive, however, since the sample size is small it is not possible to draw any formal conclusions. The assays were performed in either duplicate or triplicate dependent entirely by the number of available cells harvested. It was not possible, however, to repeat experiments with further samples due to limited availability. Following

the addition of FK506 to the SDZ RAD inhibition assays there was no consistent effect obtained, although in individual cases the ID_{50}s were altered. For example patient sample 53 there was no change in the nature of the dose response curve to SDZ RAD following the addition of FK506 at 0.1µg/l to the cultures but there was a slight additive effect at high doses of SDZ RAD. The data obtained for patient 57 revealed less inhibition of proliferation following the addition of FK506 at equimolar and at high doses of SDZ RAD. This would be consistent with previous reports investigating rapamycin and FK506 in molar excess (Dumont *et al.*, 1990 and 1994). Although the ID_{50}s were altered following the addition of FK506 there was no significant change across the group of patients tested with the mean ID_{50} for SDZ RAD alone of 25.2 µg/l and the mean ID_{50} for SDZ RAD and FK506 of 27.1µg/l.

The relatively small number of patients studied makes it difficult to formulate firm conclusions from this data. The variation of effect among individual samples within the group could imply that the mean ID_{50}s are similar simply as a mathematical quirk rather than a firm scientific observation. However, this is the data obtained using Con A stimulation of PBMC. It is not clear therefore whether the variation in the individual experiments indicates inter-experiment variation or genuine variation between individual patients. To answer this question would require repeating multiple experiments from the same donors. Unfortunately, this was not undertaken due to resource constraints.

Con A stimulated assays have been utilised throughout the experimental work described during this thesis. Although mitogen stimulation of cells is not a

physiological or specific stimulus of lymphocyte activation, it is a well-established technique to investigate the pharmacological effects of drugs on cells. By obtaining a maximal response from PBMC to mitogen stimulation any differences in the sensitivity of cells to the drugs could be detected. However, in an attempt to develop an experimental system that is more 'physiological' experiments using the MLR were performed. The potential advantage is that the MLR relies on the specific response of recipient and donor cells that differ in their histocompatibility and is therefore an *in vitro* model of the immune response to an allograft. However it is clear from the data that brisk alloresponses measured during the MLR were not always obtained. This makes the interpretation of the effects of drugs such as FK506 and SDZ RAD very difficult. The low responses seen during the MLR may have been due to the fact that the majority of donor: recipient combinations are well matched, particularly at the DR locus, which is the main drive for the MLR. This matching is obtained when selecting a patient to receive a transplant from a donor since the long-term outcome of the graft will be improved by HLA matching. During the experiments described here a surrogate donor was used as the stimulator cells during the MLR. This is not the ideal but the original donor cells were not available. Therefore, I matched surrogate donor cells to the original donor DR locus. These cells may well have behaved differently to the original donor cells had they been available but since they were matched at the DR locus then any difference would be expected to be minimal. The levels of cell stimulation obtained during the MLR experiments improved following modification of the culture medium. However, it is noted that even during the earlier experiments that the cell cultures responded well to Con A when added at 48 hours to control cultures. This suggests the cells remain viable and

capable of proliferation. Nevertheless, there are some examples where the MLR is brisk and a dose response curve is obtained to SDZ RAD and to FK506 as in patient 69 and patient 66 respectively. The effect of combining the two drugs during the MLR was variable and in particular there was no evidence of an interaction at drug levels that are equimolar. It is interesting to note that in many cases the level of cell proliferation as measured by incorporation of ^{3}H-thymidine actually initially increased following the addition of either drug at the very low doses. This phenomenon may in part be explained by the possibility of the drugs selectively inhibiting anti-proliferative cells within the cultures.

The experimental systems used to test whether there are interactions between SDZ and FK506 in lymphocytes from renal transplant recipients included both mitogen stimulation and MLR. The effects of the drugs in each experimental system tend to be the same. The data, however, do not support a consistent interaction between SDZ RAD and FK506 in *in vitro* lymphocyte cultures. This in itself does not mean that there would not be an effect *in vivo*. The reasons for the difficulties obtaining consistent data are not clear. I feel that one explanation for the variable effects seen in the experimental systems is that the cell samples on which the experiments are performed come from a heterogeneous population. By this I mean the samples were taken from patients of different ethnic backgrounds, varying levels of renal function and who had variably been exposed to powerful anti-rejection drugs. These factors may have led to a degree of background 'noise' within the experimental system. However, despite the variance the conclusions drawn from these studies are solid.

Conversely, a number of the previous reports that I have cited in this thesis have utilised either samples from healthy volunteers or from animal models. Laboratory animals can be bred to a degree of uniformity that will allow for the collection of 'clean' data. However, one is then left with the very real difficulty regarding the relevance of extrapolating the data from 'mouse to man'. Despite the limitations of these *in vitro* techniques, the pharmacodynamic assessment of individuals' lymphocyte responses to immunosuppressive drugs have been reported previously in an attempt to develop a strategy for tailoring treatment protocols. For example, the MLR in pre-operative samples from potential renal transplant patients was obtained to assess recipient sensitivities to anti-rejection drugs (Francis *et al.*, 1988). The resistant group had a higher rate of graft loss from acute rejection, more episodes of graft rejection and higher levels of serum creatinine. Sensitivity experiments performed on PBMC from subjects with renal disease including patients who had received a renal transplant report large individual variations in ID_{50} values for Cy A (Hirano *et al.*, 1997). The incidence of graft loss in those patients in the low sensitivity group compared to the highly sensitive group was significantly higher and there was an association with higher serum creatinine in the low sensitivity group. Although this is an interesting observation the numbers in each of the groups were small.

These studies attempted to predict clinical response to anti rejection drugs based on an *in vitro* test of lymphocyte sensitivity. The experiments described in this thesis utilised the *in vitro* methods of lymphocyte stimulation to test for variation in lymphocyte sensitivity to immunosuppressive drugs, for development of resistance to these drugs, and for exploring potential drug interactions between

FK506 and the structurally related novel compound, SDZ RAD. It would be valuable if an *in vitro* test of lymphocyte responsiveness could predict patients where for example the drug doses could be safely reduced. This type of work may lead to the possibility of allowing the clinician to tailor immunosuppression for individual patients.

Further experiments were performed to develop alternative assays for cell activation. Flow cytometry analyses of CD25 expression on lymphocytes from renal transplant patients and normal volunteers were performed following stimulation with Con A. Following mitogen stimulation the percentage of cells expressing CD25 was high (up to 90%) and in one patient (number 55) a dose response curve following the addition of graded doses of SDZ RAD was obtained. At the highest dose of SDZ RAD used the percentage of cells expressing CD25 fell from 90% to around 40%. In other samples the effect of SDZ RAD on expression of CD25 was less marked. There was no additional effect of combining the drug with FK506 except at high concentrations of FK506 (10μg/L) when it was most likely to be an effect of FK506 only. With the small number of experiments performed firm conclusions regarding the interaction of FK506 with SDZ RAD on expression of CD25 in lymphocyte cultures cannot be reached. From consideration of all the data relating to SDZ RAD and FK506, it is clear that they are not antagonistic when used in lymphocyte cultures from renal transplant patients.

In summary, the experiments described during this thesis have revealed some interesting and novel observations. Firstly, there is a wide range of sensitivities of PBMC from renal transplant patients to the immunosuppressant drugs, Cy A and FK506. Resistance of PBMC from renal transplant patient cells to either drug is correlated. The development of selective resistance to the drug used for treatment has potential clinical implications. If patients that are developing resistance can be predicted from similar *in vitro* studies to those reported here, then pre-emptive treatment changes could be introduced.

P-gp dependent mechanisms do not explain the variation of sensitivity to Cy A and FK506 in cell cultures from renal transplant patients. However, PBMC from all patients exhibited R-123 drug pumping and inhibition of pumping following exposure to the selective P-gp inhibitor, PSC 833. This implies that P-gp dependent drug pumping occurs in PBMC from renal transplant patients.

The combination of FK506 and the rapamycin analogue, SDZ RAD did not show any antagonism in *in vitro* PBMC cultures. Immunosuppressant protocols continue to be refined and future protocols may contain SDZ RAD in combination with FK506.

Lastly, I have derived much pleasure from investigating and presenting these fascinating areas of research that combine medicine and science.

APPENDIX 1

Information and Consent Form

Title of project

Characterisation of Multidrug-Resistance Phenotypes in T-lymphocytes from Renal Transplant Patients.

Description

We would like you to help us with some research. We would like to take some extra blood at the time of routine blood-taking. White blood cells (the type that cause rejection) will be extracted and studied for their responses to anti-rejection drugs. The additional amount of blood required is a maximum of 20mls. And we may ask you to help us more than once with this.

The aim of the study is to see if we can identify resistance to anti-rejection drugs and explore potential resistance mechanisms. If this were to be the case , we could plan anti-rejection treatment in the future to try and avoid transplant rejection, rather than just treat it when it occurs.
The research is at an early stage, and we cannot use these results to plan your drug treatment. Therefore your drug treatment will not be affected by this study.

You are free to spend some free time thinking about this study, and to discuss it with either Dr Higgins or Dr Delaney. Even if you agree to take part, you are quite free to change your mind at any time without giving a reason. Taking part in the study will not affect your treatment in any way.

Thank you for your attention.

Potential risks of research: those of taking a slightly larger blood sample from you.

Potential benefit of the research: improving future anti-rejection drug treatment.

Name of Investigators: Dr. Mike Delaney and Dr. Rob Higgins

Name of research volunteer:
I have read the description of the research project given above and the investigator has explained it to me to my satisfaction. I am aware that there may be some risk attached, and that my should I suffer injury as a consequence of the research the University of Warwick will pay compensation. The level of the compensation will be determined by reference to damages awarded by courts for similar injuries at the time the injury was suffered.

I understand that I may withdraw from the project at any time for any reason and such a withdrawal will in no way affect the treatment I am receiving.

Signed (Volunteer):

Signed (Investigator):

APPENDIX II

Publications and Abstracts

Delaney, M. P., Smythe, E., Higgins, R. M., and Morris, A. G. (2000). Constitutive and acquired resistance to calcineurin inhibitors in renal transplantation: role of P-glycoprotein-170. *Transplant International* **13, 276-284.**

Delaney, M.P., Higgins, R.M. and Morris, A.G. (2001) FK506 And The Rapamycin Analogue, SDZ RAD Do Not Have Antagonistic Effects On Lymphocytes From Renal Transplant Patients. *American Society of Transplantation (AST)* Abstract # 657

Delaney, M. P., Smythe, E., Higgins, R. M., and Morris, A. G. (1999). Sensitivity Of Lymphocytes To Cyclosporin A And Tacrolimus Is Not Mediated By P-Glycoprotein-170. *The International Society of Nephrology (ISN)*

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